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(54) Title: REGULATION OF INSULIN PRODUCTION

(57) Abstract: Gene therapy-based treatment of type-2 diabetes, obesity, and related conditions by *in vivo* expression of glucagon-like peptide 1 (GLP-1), and/or glucose-dependent insulinotropic peptide (GIP). The treatment may be combined with concurrent administraton of dipeptidyl peptidase IV (DPP-IV) inhibitors.

REGULATION OF INSULIN PRODUCTION

This application claims the benefit under 35 USC §119(e) of United States Provisional Patent Application No. 60/328,116, filed October 9, 2001, for "Regulation of Insulin Production in a Mammal." The disclosure of this application is incorporated herein by reference in its entirety.

Background of the Invention

The invention provides gene therapy-based treatment of type-2 diabetes, obesity, and related conditions by in vivo expression of glucagon-like peptide 1 (GLP-1), and/or glucose-dependent insulinotropic peptide (GIP). The treatment may be combined with concurrent administration of dipeptidyl peptidase IV (DPP-IV) inhibitors.

Type-2 diabetes is characterized by hyperglycemia, hyperinsulinemia, and hyperlipidemia. Impaired insulin release from pancreatic β-cells and insulin resistance in peripheral tissues (skeletal muscle and adipose tissues) results in impaired peripheral glucose disposal and causes hyperglycemia. Insulin resistance causes a compensatory increase in insulin secretion from pancreatic β-cells, which results in hyperinsulinemia. Increased fatty acid flux to the liver, caused by insulin resistance, increases lipoprotein production and results in hyperlipidemia. Therefore, a mechanism to allow glucose-induced insulin secretion would provide an improved therapy for type 2 diabetes.

GLP-1 is a peptide synthesized in intestinal L cells by proteolytic cleavage of the preproglucagon molecule. Functions of GLP-1 include the enhancement of regulated secretion of insulin from pancreatic β-cells in response to increased blood glucose levels and suppression of glucagon secretion, which together results in a decrease in blood glucose levels without causing hypoglycemia (Thorens, 1995; Kieffer and Habener, 2000). Other functions of GLP-1 include delayed gastric emptying and a reduction in appetite and food intake (Gutzwiller et al., 1999; Flint et al., 1998; 2000).

GLP-1 has an extremely short half-life in vivo (<2 minutes). In man, GLP-1 is quickly inactivated by DPP-IV. Therefore, much effort has been placed on the development of GLP-1 analogues resistant to DPP-IV degradation (Burcelin et al., 1999; Gallwitz et al., 2000), as well as natural GLP-1 analogues isolated from other species such as exendin 4 (Hughes, 1998; Greig et al., 1999; Young et al., 1999). Alternatively, others have focused on

the development of small molecule inhibitors of DPP-IV, which would inhibit GLP-1 degradation and functionally increase the half-life of GLP-1 (Holst and Deacon, 1998).

Infusion of GLP-1 peptide into diabetic patients results in normalization of blood glucose levels (Nauck et al., 1993; 1998; Habener, et al. 1993; Rachman et al., 1997) and GLP-1 administration has been demonstrated to reduce food intake in humans (Flint et al., 1998; Gutzwiller et al., 1999). Therefore, it may play a role in controlling satiety. These data suggest that GLP-1 is a potentially therapeutic agent for use in improving β-cell function and glycemic control in type 2 diabetes.

GIP, a peptide synthesized by duodenum K cells, functions to stimulate insulin release in response to increased blood glucose levels and may also have the advantage of lowering blood lipid levels (Kieffer and Habener, 2000). Unlike GLP-1, GIP has not been demonstrated to improve the phenotype of diabetic patients (Service et al., 1990), although GIP has been shown to enhance insulin-mediated glucose disposal in sheep (Rose et al., 1988), rats (O'Harte et al., 1999) and mice (O'Harte et al., 2000). Therefore, the expression of GIP in combination with GLP-1 may provide an improved therapy that surpasses the effect of each peptide alone. Finally, a recent study has demonstrated that DPP-IV inhibition reduces GIP degradation and potentiates its insulinotropinic and antihyperglycemic effects in pigs (Deacon et al., 2001).

A significant limitation to potential GLP-1 and GIP therapy in diabetic patients is the short biological half-life that makes protein replacement therapy problematic. In addition, the endogenous expression of GLP-1 and GIP, combined with the use of a small molecule DPP-IV inhibitor, will increase the circulating levels of both hormones and add a means to control GLP-1 and GIP steady-state levels. The insulinotropic hormones, GLP-1 and GIP, are metabolized rapidly by the ubiquitous enzyme, DPP-IV, resulting in the formation of an inactive, or, in some cases, an antagonistic peptide. DPP-IV has a dual function as a regulatory protease and as a binding protein. DPP-IV cleaves Xaa-Pro or Xaa-Ala dipeptides from the N-terminus of regulatory peptides. This minimal truncation of peptides by DPP-IV results in inactivation of many mammalian regulatory peptides including: neuropeptides such as neuropeptide Y or endomorphin, circulating peptide hormones like peptide YY, growth hormone-releasing hormone, GLP-1, and GLP-2, GIP, and many others (reviewed by Mentelin, 1999). Due to this rapid degradation, the effects of single, bolus protein administration of GLP-1 and/or GIP peptide are short lasting, and for demonstration of the

peptide antidiabetogenic effects, continuous intravenous infusion is required. Therefore, constant level, endogenous secretion of these incretin hormones may provide an advantage over protein replacement therapy. This constant level endogenous secretion can be achieved by in vivo expression of GLP-1 and/or GIP-1.

Gutless adenoviral vectors have been shown to achieve high-level transgene expression and improved duration of expression with reduced vector toxicity. Such vectors also have a large coding capacity (up to 38 kb) and can encode multiple transgene expression cassettes including ligand-regulated transcriptional control elements (Kochanek, 1999).

Retroviral vectors and lentiviral viral vectors have a coding capacity of approximately 8 kb, and the viral genome is capable of integration into the host cell chromosome, thus allowing for potentially life-long transgene expression. One non-limiting example of a lentivirus is Bovine-Immunodeficiency-Virus (BIV). (WO 01/44458).

AAV vectors integrate into the host genome and such vectors are capable of long-term transgene expression. The coding capacity of AAV vectors is approximately 5 kb.

Summary of the Invention

In view of the above, the present invention provides the following exemplary enumerated embodiments:

1. A viral vector comprising:

(a) a polynucleotide sequence encoding GLP1;

(b) a polynucleotide sequence encoding a signal sequence upstream of (a); and

(c) a polyadenylation signal downstream of (a).

2. The vector according to embodiment 1, further comprising a polynucleotide sequence encoding a proteolytic cleavage site located between said polynucleotide sequence encoding GLP1 and said polynucleotide sequence encoding the signal sequence.

3. The vector according to embodiment 1 or 2, wherein GLP1 comprises SEQ ID NO:2, 4, 6, 8, or 10.

4. The vector according to embodiment 1 or 2, wherein said polynucleotide sequence encoding GLP1 comprises SEQ ID NO:1, 3, 5, 7 or 9.

5. The vector according to any of embodiments 1-4, wherein said signal sequence is an IgK signal sequence.

6. The vector according to embodiment 5, wherein said IgK signal sequence comprises SEQ ID NO:18.

7. The vector according to embodiment 6, wherein said polynucleotide encoding the IgK signal sequence comprises SEQ ID NO:17.

8. The vector according to any of embodiments 1-4, wherein said signal sequence is an exendin signal sequence.

9. The vector according to embodiment 8 wherein said exendin signal sequence comprises SEQ ID NO:16.

10. The vector according to embodiment 9, wherein said polynucleotide encoding the exendin signal sequence comprises SEQ ID NO:15.

11. The vector according to any one of the preceding embodiments, wherein the polyadenylation signal is derived from SV40.

12. The vector according to any one of embodiments 2-11, wherein said proteolytic cleavage site is cleaved by furin protease.

13. The vector according to any one of the preceding embodiments, wherein expression of said polynucleotide sequence encoding GLP1 is controlled by a regulatable promoter.

14. The vector according to any of embodiments 1-13, wherein said vector is an adenoviral vector.

15. The vector according to any of embodiments 1-13, wherein said vector is a retroviral vector.

16. The vector according to any of embodiments 1-13, wherein said vector is a lentiviral vector.

17. The vector according to any of embodiments 1-13, wherein said vector is an adeno associated viral vector.

18. The vector according to any one of the preceding embodiments, further comprising a polynucleotide encoding GIP.

19. The vector according to embodiment 18, wherein GIP comprises SEQ ID NO:14.

20. The vector according to embodiment 19, wherein said polynucleotide sequence encoding GIP comprises SEQ ID NO:13.

21. A mammalian cell comprising the vector according to any one of embodiments 1-20.

22. A packaging cell comprising the vector according to any one of embodiments 1-20.

23. A method of making a viral particle comprising culturing the packaging

cell of embodiment 22 under conditions wherein the particle is produced.

24. A method of treating diabetes in a mammal comprising administering a physiologically effective amount of the vector in any one of embodiments 1-20 to the mammal.

25. The method of embodiment 24, wherein the mammal is a primate.

26. The method of embodiment 25, wherein the primate is a human.

27. The method of treating diabetes according to any one of embodiments 24-26, further comprising administering a DPP-IV inhibitor to said mammal.

28. A viral vector comprising:

(a) a polynucleotide sequence encoding GIP;

(b) a polynucleotide sequence encoding a signal sequence upstream of (a); and

(c) a polyadenylation signal downstream of (a).

29. The vector according to embodiment 27, further comprising a polynucleotide sequence encoding a proteolytic cleavage site located between said polynucleotide sequence encoding GIP and said polynucleotide sequence encoding the signal sequence.

30. The vector according to embodiment 28 or 29, wherein GIP comprises SEQ ID NO:14.

31. The vector according to embodiment 30, wherein said polynucleotide sequence encoding GIP comprises SEQ ID NO:13.

32. The vector according to any of embodiments 28-31, wherein said signal sequence is an IgK signal sequence.

33. The vector according to embodiment 32, wherein said IgK signal sequence comprises SEQ ID NO:18.

34. The vector according to embodiment 33, wherein said polynucleotide encoding the IgK signal sequence comprises SEQ ID NO:17.

35. The vector according to any of embodiments 28-31, wherein said signal sequence is an exendin signal sequence.

36. The vector according to embodiment 35, wherein said exendin signal sequence comprises SEQ ID NO:16.

37. The vector according to embodiment 36, wherein said polynucleotide encoding the exendin signal sequence comprises SEQ ID NO:15.

38. The vector according to any one of embodiments 28-37, wherein the polyadenylation signal is derived from SV40.

39. The vector according to any one of embodiments 29-38, wherein said proteolytic cleavage
site is cleaved by furin protease.

40. The vector according to any one of embodiments 28-39, wherein expression of said polynucleotide sequence encoding GIP is controlled by a regulatable promoter.

41. The vector according to any of embodiments 28-39, wherein said vector is an adenoviral vector.

42. The vector according to any of embodiments 28-39, wherein said vector is a retroviral vector.

43. The vector according to any of embodiments 28-39, wherein said vector is a lentiviral vector.

44. The vector according to any of embodiments 28-39, wherein said vector is an adeno associated viral vector.

45. The vector according to any one of embodiments 28-44, further comprising a polynucleotide sequence encoding GLP1.

46. A mammalian cell comprising the vector according to any one of embodiments 28-45.

47. A packaging cell comprising the vector according to any one of embodiments 28-45.

48. A method of making a viral particle, comprising culturing the packaging
cell according to embodiment 47 under conditions wherein the particle is produced.

49. A method of treating diabetes in a mammal, comprising administering a physiologically effective amount of the vector according to any one of embodiments 28-45 to the mammal.

50. The method of embodiment 49, wherein the mammal is a primate.

51. The method of embodiment 50, wherein the primate is a human.

52. The method of treatment as specified in any one of embodiment 49-51, further comprising
administering a DPP-IV inhibitor to said mammal.

Brief Description of the Drawings

Figure 1A Map of plasmid pCiGLP1IgK+Ala

Figure 1B Map of plasmid pCiGLP1Ext

Figure 1C Map of plasmid pCi-nGLP1IgkplfHis

Figure 2 Adenoviral vectors encoding Tet system

Figure 3A Map of plasmid pGTL14GLP+Ala

Figure 3B Map of plasmid pGTL15GLP+Ala

Figure 3C Map of plasmid pGTL146GLPex

Figure 3D Map of plasmid pGTL15GLPex

Figure 3E Map of plasmid pGTI.14GLPIgKplf

Figure 3F Map of plasmid pGTI.15GLPIgKplf

Figure 4A Map of plasmid pBV2.CGE.C7.GLP-1Ex

Figure 4B Map of plasmid pAGVC7endo1c

Figure 4C Map of plasmid pGTL24aPL2

Figure 4D Map of plasmid pGTIAPL2.CGE.C7.GLP-1Ex

Figure 4E Map of plasmid pbv2

Figure 5A Map of plasmid pBV2.CMV.GLP-1Ex

Figure 5B Map of plasmid pGTLAPL2.CMV.GLP-1Ex

Figure 6A Map of plasmid pGTI.APL2.ALB.GLP-1Ex

Figure 6B Map of plasmid pBV2.ALB.GLP-1Ex

Detailed Description of the Invention

The practice of the present invention employs, unless otherwise indicated, conventional techniques of chemistry, molecular biology, microbiology, recombinant DNA, genetics, immunology, cell biology, cell culture and transgenic biology, which are within the skill of the art. See, e.g., Maniatis et al., 1982; Sambrook et al., 1989; Sambrook and Russell, 2001; Ausubel et al., 1992; Glover, 1985; Anand, 1992; Guthrie and Fink, 1991; Harlow and Lane, 1988; Jakoby and Pastan, 1979; Nucleic Acid Hybridization (B. D. Hames & S. J. Higgins eds. 1984); Transcription And Translation (B. D. Hames & S. J. Higgins eds. 1984); Culture Of Animal Cells (R. I. Freshney, Alan R. Liss, Inc., 1987); Immobilized Cells And Enzymes (IRL Press, 1986); B. Perbal, A Practical Guide To Molecular Cloning (1984); the treatise, Methods In Enzymology (Academic Press, Inc., N.Y.); Gene Transfer Vectors For

Mammalian Cells (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); Methods In Enzymology, Vols. 154 and 155 (Wu et al. eds.), Immunochemical Methods In Cell And Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); Handbook Of Experimental Immunology, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); Riott, Essential Immunology, 6th Edition, Blackwell Scientific Publications, Oxford, 1988; Hogan et al., Manipulating the Mouse Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

As used in this specification and claims, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, a virion particle includes a plurality of virion particles.

In one embodiment of the invention, a nucleic acid or multiple nucleic acids encoding GLP-1 and/or GIP-1 are delivered to a cell, causing the cell to express increased amounts of GLP-1 and/or GIP-1. The cells of this embodiment may either be cultured *in vivo* or *in vitro* and transduced with nucleic acids encoding GLP-1 and/or GIP-1. Preferably, the cell is eukaryotic; more preferably, mammalian; and most preferably, human. This cell of the invention when in vitro may be used to produce GLP-1 and/or GIP-1 protein. These protein(s) may be further purified for delivery to an animal or for further studies related to the effects of GLP-1 and/or GIP-1 on cells, including their regulating effects on insulin production. The purified proteins may be delivered to an animal to study their regulating effects on insulin production in the animal. Preferably, the animal is a mammal and most preferably, a human.

Preferably, nucleic acids encoding GLP-1 and/or GIP-1 are delivered to a cell via a gene delivery vector. Gene delivery vectors include, but are not limited to, plasmids, phage, cosmids or viral based vectors.

As used herein, the term "viral vector" is used according to its art-recognized meaning. The viral vectors of the invention may be utilized for the purpose of transfering DNA, RNA or other nucleic acids into cells either in vitro or in vivo. Viral vectors include, but are not limited to, retroviral vectors, vaccinia vectors, lentiviral vectors, herpes virus vectors (e.g., HSV), baculoviral vectors, cytomegalovirus (CMV) vectors, papillomavirus vectors, simian virus (SV40) vectors, Sindbis vectors, semliki forest virus vectors, adenoviral vectors, and adeno-associated viral (AAV) vectors.

One embodiment of the invention includes vectors encoding GLP-1 and/or GIP. The vectors can be used to deliver GLP-1 and/or GIP encoding sequences to cells in vitro or in vivo. Thus, these vectors are useful for expression of GLP-1 and/or GIP in vitro or in vivo. Another embodiment of the invention includes cells containing the vectors of the invention.

The invention also comprises polynucleotides that encode the proteins of the invention. As used herein, the term "polynucleotide" means a nucleic acid molecule, such as DNA or RNA, that encodes a polypeptide. The molecule may include regulatory sequences. Preferably, the polynucleotide is DNA. Such polynucleotides are prepared or obtained by techniques known by those skilled in the art in combination with the teachings contained therein. The polynucleotides of the invention also include polynucleotides that differ in certain bases but still encode the proteins of the invention due to the redundancy of the genetic code.

The genes of the invention can be optimized for enhanced expression in the organism of choice. In this manner, the genes can be synthesized utilizing preferred codons for the organism of choice. That is the most preferred codon for a particular host is the single codon, which most frequently encodes that amino acid in that host. For example, the human preferred codon for a particular amino acid may be derived from known gene sequences from humans. Synthetic genes can also be made based on the distribution of codons a particular host uses for a particular amino acid. It is understood that optimization of a gene does not require every codon encoding for a particular amino acid be changed to the most frequently used codon in that particular organism. Optimization requires at least one codon encoding for a particular amino acid be changed to a codon which is more frequently used in that organism for the same amino acid. It does not need to be the most frequently used codon. Preferably more than 5% of the codons are changed to an equivalent codon that is used more frequently in the particular organism. More preferably more than 10% of the codons are changed to an equivalent codon that is used more frequently in the particular organism. Most preferably more than 20% of the codons are changed to an equivalent codon that is used more frequently in the particular organism. By equivalent codon is meant that the codon encodes for the same amino acid in the organism.

In this manner, the nucleotide sequences can be optimized for expression in any organism. It is recognized that all or any part of the gene sequence may be optimized or synthetic. That is, synthetic or partially optimized sequences may also be used.

Recoding of a gene or portions of a gene can be performed using techniques well known in the art. By way of non-limiting examples, Casimiro DR et al. describes a PCR-based method for gene synthesis(Structure 1997 Nov 15;5(11):1407-12) (See also Brocca et al. "Design, total synthesis, and functional overexpression of the Candida rugosa lip1gene coding for a major industrial lipase" Protein Sci 1998 Jun;7(6):1415-22; Withers-Martinez C, et al., "PCR-based gene synthesis as an efficient approach for expression of the A+T-rich malaria genome" Protein Eng 1999 Dec;12(12):1113-20; and Stemmer et al., "Single-step assembly of a gene and entire plasmid from large numbers of oligodeoxyribonucleotides" Gene 1995 Oct 16;164(1):49-53).

A functional variant may differ in amino acid sequence by one or more substitutions, additions, deletions, truncations which may be present in any combination, but would retain the same biological function as the reference gene (see Biological function). The present invention includes vectors expressing a functional variant of GLP-1 and/or GIP.

"Biological function" within the meaning of this application is to be understood in a broad sense. It includes, but is not limited to, the particular functions of the GLP-1 and/or GIP protein disclosed in this application. Thus, biological functions are not only those which a polypeptide displays in its physiological context, i.e. as part of a living organism or cell, but includes functions which it may perform in a non-physiological setting, e.g. in vitro. For example, a biological function of the GLP-1 and/or GIP protein within the meaning of this application is the ability to, for example, demonstrate regulation of insulin production either *in vitro* or *in vivo*. Assays to assess the required properties are well-known in the art.

"Polynucleotide" and "nucleic acid", used interchangeably herein, generally refers to any polyribonucleotide or polydeoxyribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. "Polynucleotides" include, without limitation single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, RNA that is a mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single- stranded or more typically double stranded or a mixture of single- and double-stranded regions. In addition polynucleotide refers to triple- stranded regions comprising RNA or DNA or both RNA and DNA. "Polynucleotide" also includes a polymer comprising purine and pyrimidine bases, or other natural, chemically, biochemically modified, non-natural or derivatized nucleotide bases. The term polynucleotide also includes DNAs or RNAs containing one or more modified bases

and DNAs or RNAs with backbones modified for stability or for other reasons. The backbone of the polynucleotide can comprise sugars and phosphate groups (as may typically be found in RNA or DNA), or modified or substituted sugar or phosphate groups. "Modifed bases" includes for examples tritylated bases and unusual bases such as inosine. A variety of modifications have been made to DNA and RNA; thus "polynucleotide" embraces chemically, enzymatically or metabolically modified forms of polynucleotides as typically found in nature as well as the chemical forms of DNA and RNA characteristic of viruses and cells. "Polynucleotide" also embraces relatively short polynucleotides, often referred to as oligonucleotides. As used herein, "DNA" includes not only bases A, T, C, and G, but also includes any of their analogs or modified forms of these bases, such as methylated nucleotides, internucleotide modifications such as uncharged linkages and thioates, use of sugar analogs, and modified and/or alternative backbone structures, such as polyamides. The term "DNA" also includes cDNA, genomic DNA or synthetic DNA.

The terms "promoter", "promoter region", or "promoter sequence" refer generally to transcriptional regulatory regions of a gene, which may be found at the 5' or 3' side of the coding region, or within the coding region, or within introns. Typically, a promoter is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. The typical 5' promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence is a transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

The invention includes the use of hybrid GLP-1 and/or GIP nucleotide sequences that contains a heterologous promoter, a heterologous signal sequence (to allow secretion), the coding region, and a poly(A) signal. This nucleotide sequence is encoded in a gene delivery vector. Preferable vectors include an adenovirus (preferably a "gutless" adenovirus), AAV, or lentiviral (preferably BIV) based vectors. Preferably, the promoter is a regulated promoter and transcription factor expression system, such as the published tetracycline-regulated systems, or other regulatable systems (WO 01/30843), to allow regulated expression of GLP-1 incorporated into gutless adenoviral or lentiviral vectors. Preferably, the vector is targeted to a specific cell type. Preferably the cell is a hepatocyte.

The term "adenoviral particle" is to be understood broadly as meaning infectious viral particles that are formed when an adenoviral vector of the invention is transduced into an appropriate cell or cell line for the generation of infectious particles. As used herein, the term "adenovirus" or "adenoviral particle" is used to include any and all viruses that may be categorized as an adenovirus, including any adenovirus that infects a human or an animal, including all groups, subgroups, and serotypes. Preferably, such adenoviruses are ones that infect human cells. Such adenoviruses may be wild-type or may be modified in various ways known in the art or as disclosed herein. Such modifications include modifications to the adenovirus genome that is packaged in the particle in order to make an infectious virus. Such modifications include deletions known in the art, such as deletions in one or more of the E1, E2a, E2b, E3, or E4 coding regions. Such modifications also include deletions of all of the coding regions of the adenoviral genome. Such adenoviruses are known as "gutless" adenoviruses. The terms also include replication-conditional adenoviruses; that is, viruses that replicate in certain types of cells or tissues but not in other types. These include the viruses disclosed in U.S. Patent No. 5,998,205, issued December 7, 1999 to Hallenbeck et al. and U.S. Patent No. 5,801,029, issued September 1, 1998 to McCormick, the disclosures of both of which are incorporated herein by reference in their entirety. Such viruses are sometimes referred to as cytolytic or cytopathic viruses (or vectors), and, if they have such an effect on neoplastic cells, are referred to as oncolytic viruses (or vectors).

In another embodiment, adenoviral vectors of the present invention are delivered by intravenous injection (IV) to an animal. Preferably the animal is a mammal; more preferably a primate and most preferably a human. These adenoviral vectors may be delivered at a dose of about $1x10^9$ to $5x10^{13}$ adenoviral vector particles per kilogram (kg); preferably at a dose of about $1x10^{10}$ to $1x10^{13}$ adenoviral vector particles per kg; and most preferably for replicating vectors (e.g. "oncolytic" vectors) about $5x10^{10}$ to $5x10^{11}$ and for replication defective vectors about $1x10^{10}$ to $1x10^{13}$ adenoviral vector particles per kg.

The adenoviral vectors of the invention are made by standard techniques known to those skilled in the art. The vectors are transferred into packaging cells by techniques known to those skilled in the art. Packaging cells provide complementing functions to the functions provided by the genes in the adenovirus genome that are to be packaged into the adenovirus particle. The production of such particles requires that the vector be replicated and that those proteins necessary for assembling an infectious virus be produced. The packaging cells are

cultured under conditions that permit the production of the desired viral vector particle. The particles are recovered by standard techniques. The preferred packaging cells are those that have been designed to limit homologous recombination that could lead to wild-type adenoviral particles. Such cells are disclosed in U.S. Patent Nos. 5,994,128, issued November 30, 1999 to Fallaux, et al., and 6,033,908, issued March 7, 2000 to Bout, et al. The packaging cell known as PER.C6, which is disclosed in these patents, is particularly preferred.

Lentiviral and retroviral vectors are generally constructed such that the majority of the viral genes are deleted and replaced by a gene of interest. Most frequently the gene of interest is transcribed under the control of the viral regulatory sequences within the long terminal repeat (LTR). Alternatively, the gene of interest may be expressed under the regulation of its own internal promoter. The genes which have been deleted from the vector are generally provided by one or more helper or packaging constructs in a packaging cell line (Bender et al., J. Virol. 61: 1639 – 1649 (1987) and Miller et al., Biotechniques, 7:980 –990 (1989)). Also see Markowitz et al., J. Virol. 62:1120 – 1124 (1988) wherein complementary portions of the helper construct were divided on two separate constructs. The packaging cell line may be transfected with the vector, thereby producing vector RNA that is packaged into the virus particles. These released virus particles are replication defective and can be used to deliver the vector carrying the gene of interest to target cells. Preferably, the vector is derived from a lentivirus and preferably, from BIV.

To increase safety, efficiency and accuracy of the recombinant vector systems, various improved recombinant systems have been constructed. One type of improvement includes making safer packaging cell lines that are generated by deletions in the 3' Long Terminal Repeat (LTR). Other improvements include increasing the host range by replacement of one viral env gene with that of another viral env gene thereby creating a hybrid producer line that generates pseudotyped helper viruses. More specifically HIV has been given an extended host cell range by pseudotyping with the unrelated viruses VSV and HSV (Zhu et al., J. Aids, 3:215 – 219 (1990) and Naldini et al., Science, 272:263-267, (1996)). Further improvements have been made by the use of minimum viral coding regions on the vector. Lentivirus can infect nondividing cells and this property is especially useful for gene delivery. Additionally, most packaging cell lines currently in use have been transfected with separate plasmids each containing one of the necessary coding sequences so that

multiple recombination events would be necessary before replication competent virus can be produced. PCT publication WO 01/44458, which is hereby incorporated by reference, describes examples of a BIV based lentiviral vector system.

In another embodiment, treatment of type-2 diabetes, obesity, and related conditions is achieved by in vivo expression of glucagon-like peptide 1 (GLP-1), and/or glucose-dependent insulinotropic peptide (GIP), preferably combined with concurrent administration of DPP-IV inhibitors. GLP-1, GIP, or a combination of both GLP-1 and GIP are delivered to the animal via gene delivery vectors. These vectors are used to induce insulin secretion and can be used to study diabetes, obesity and related diseases. These vectors may also be used to treat diabetes, obesity and related diseases. Preferable vectors include adenoviral vectors (preferably lacking all viral genes, i.e. high capacity or gutless), lentiviral vectors (e.g. HIV, BIV-based), and adeno-associated virus (AAV) vectors.

Signal sequences can be used in the GLP-1 or GIP expression cassette. Two preferred signal sequences are the murine Igκ signal and the glia monster exendin signal (Chen and Drucker, 1996). In addition, because GLP-1 and GIP are such small peptides (less than 40 aa), to achieve efficient translation, signal cleavage, and secretion, it is preferable to lengthen the pro-peptide. This can be achieved by the addition of amino acids at the amino terminal, downstream of the signal peptide. The pro-sequence can be specifically cleaved from the GLP-1 or GIP coding region by a protease. A preferred protease is furin, when a furin cleavage site is engineered into the pro-sequence.

One embodiment has the expression of GLP-1 and/or GIP controlled by a constitutive promoter. Non-limiting examples of constitutive promoters include the cytomegalovirus (CMV) promoter, the Rous sarcoma long- terminal repeat (LTR) sequence, and the SV40 early gene promoter.

Another embodiment has the expression of GLP-1 and/or GIP controlled by an inducible promoter. One example of a controlled promoter system is the Tet-On ™ and Tet-Off ™ systems currently available from Clontech (Palo Alto, CA). This promoter system allows the regulated expression of the transgene controlled by tetracycline or tetracycline derivatives, such as doxycycline. This system could be used to control the expression of GLP-1 and/or GIP. Other regulatable promoter systems are described in the PCT publications WO 01/30843 and WO 02/06463, which are hereby incorporated by reference in their entirety.

A significant limitation to potential GLP-1 and GIP therapy in diabetic patients is the short biological half-life that makes protein replacement therapy problematic. The short half-life is due to the cleavage of GLP-1 and GIP by DPP-IV. Two alternative strategies have been employed to circumvent this problem. For GLP-1, variants derived from different species, such as the glia monster exendin peptide (Hughes, 1998; Grieg et al., 1999; Young et al., 1999), or derivatives of the mammalian GLP-1, that are not readily degraded by DPP-IV (Burcelin et al., 1999; Gallwitz et al., 2000), have been generated and characterized.

Another embodiment of the invention includes vectors including GLP-1 variants that have increased resistance to DPP-IV degradation. These variants include, but are not limited to, those known in the art. For example, those described by Hughes (1998); Grieg et al. (1999); and Young et al., (1999).

Alternatively, an approach to inhibit the action of DPP-IV in vivo, thereby functionally increasing the half-life of GLP-1 and/or GIP, has been employed (reviewed by Holst and Deacon, 1998). It is not expected that inhibition of DPP-IV would have long-term negative or toxic effects on the treated patient, since DPP-IV-deficient rats appear completely unaffected by this deficiency (Pederson et al., 1996). Animal studies have been reported demonstrating that administration of DPP-IV inhibitors, such as isoleucine thiazolidide or valine-pyrrolidide, leads to an improvement in glucose tolerance (Pederson et al., 1998, Pauly et al., 1999; Deacon et al., 2001). The demonstration of the utility of these compounds has been described (Balkan et al., 1999). Non-limiting examples of DPP-IV inhibitors are described in US 6,110,949, US 6,107,317, US 6,011,155, US 6,172,081, and US 6,166,063, which are hereby incorporated by reference in their entirety.

"GLP1" is defined as GLP1(7-37), GLP1(7-36), or variants thereof.

"Expression cassette" is defined as a polynucleotide, when introduced to the desired cell, will express the encoded amino acid.

"Secretion signal" is defined as an amino acid sequence, or an amino acid sequence encoded by a polynucleotide, which, when present at the amino terminus of a polypeptide, functions to mediate polypeptide secretion.

"Administration" is defined as in vivo delivery of the gene transfer vehicle.

"Gene delivery vector" is defined as a gene transfer vehicle capable of transferring the gene of interest following in vivo or in vitro administration.

"Exendin gene" is defined as any nucleotide encoding for the glia monster exendin peptide (Seq ID:12), including SEQ ID:11.

Furin cleavage site is defined as the sequence Arg-Xaa-Lys-Arg (SEQ ID:18), where Xaa is preferentially Thr.

"Igk gene" is defined as the immunoglobulin kappa gene, including the signal sequence.

"Adenovirus vector" or "adenoviral vector" (used interchangeably) is defined as a polynucleotide comprising all or a portion of an adenoviral genome.

Table 1

GLP-1 peptides and derivatives

GLP-1 (7-37) (SEQ ID NO:1 and 2)

5'-CAC-GCT-GAA-GGG-ACC-TTT-ACC-AGT-GAC-GTA-AGT-TCT-TAT-TTG-GAA-GGC-
H A E G T F T S D V S S Y L E G

CAA-GCT-GCC-AAG-GAA-TTC-ATT-GCT-TGG-CTG-GTG-AAA-GGC-CGA-GGA-3'
Q A A K E F I A W L V K G R G

GLP-1 (7-36) (SEQ ID NO:3 and 4)

5'-CAC-GCT-GAA-GGG-ACC-TTT-ACC-AGT-GAC-GTA-AGT-TCT-TAT-TTG-GAA-GGC-
H A E G T F T S D V S S Y L E G

CAA-GCT-GCC-AAG-GAA-TTC-ATT-GCT-TGG-CTG-GTG-AAA-GGC-CGA-3'
Q A A K E F I A W L V K G R

GLP-1 Variants

GLP-1 (7-37) (A-8-G) (SEQ ID NO:5 and 6)

5'-CAC-GGT-GAA-GGG-ACC-TTT-ACC-AGT-GAC-GTA-AGT-TCT-TAT-TTG-GAA-GGC-
H G E G T F T S D V S S Y L E G

CAA-GCT-GCC-AAG-GAA-TTC-ATT-GCT-TGG-CTG-GTG-AAA-GGC-CGA-GGA-3'
Q A A K E F I A W L V K G R G

GLP-1 (7-37) (A-8-S) (SEQ ID NO:7 and 8)

5'-CAC-TCT-GAA-GGG-ACC-TTT-ACC-AGT-GAC-GTA-AGT-TCT-TAT-TTG-GAA-GGC-
H S E G T F T S D V S S Y L E G

CAA-GCT-GCC-AAG-GAA-TTC-ATT-GCT-TGG-CTG-GTG-AAA-GGC-CGA-GGA-3'
Q A A K E F I A W L V K G R G

GLP-1 (7-37) (A-8-T) (SEQ ID NO:9 and 10)

5'-CAC-ACT-GAA-GGG-ACC-TTT-ACC-AGT-GAC-GTA-AGT-TCT-TAT-TTG-GAA-GGC-
H T E G T F T S D V S S Y L E G

CAA-GCT-GCC-AAG-GAA-TTC-ATT-GCT-TGG-CTG-GTG-AAA-GGC-CGA-GGA-3'
Q A A K E F I A W L V K G R G

Table 2

Exendin Nucleotide And Amino Acid Sequence (SEQ ID NO:11 and 12)

5'-CAT-GGT-GAA-GGA-ACA-TTT-ACC-AGT-GAC-TTG-TCA-AAA-CAG-ATG-GAA-
H G E G T F T S D L S K Q M E

GAG-GAG-GCA-GTG-CGG-TTA-TTT-ATT-GAG-TGG-CTT-AAG-AAC-GGA-GGA-CCA
E E A V R L F I E W L K N G G P

AGT-AGC-GGG-GCA-CCT-CCG-CCA-TCG-3'
S S G A P P P S

GIP Nucleotide And Amino Acid Sequence (SEQ ID NO:13 and 14)

5'-TAC-GCG-GAA-GGG-ACT-TTC-ATC-AGT-GAC-TAC-AGT-ATT-GCC-ATG-GAC-
Y A E G T F I S D Y S I A M D

AAG-ATT-CAC-CAA-CAA-GAC-TTT-GTG-AAC-TGG-CTG-CTG-GCC-CAA-AAG-GGG-
K I H Q Q D F V N W L L A Q K G

AAG-AAG-AAT-GAC-TGG-AAA-CAC-AAC-ATC-ACC-CAG-3'
K K N D W K H N I T Q

Table3

Signal Sequences

Exendin signal (SEQ ID NO:15 and 16)

5'-ATG-AAA-ATC-ATC-CTG-TGG-CTG-TGT-GTT-TTT-GGG-CTG-TTC-CTT-GCA-ACT-
M K I I L W L C V F G L F L A T

TTA-TTC-CCT-ATC-AGC-TGG-CAA-ATG-CCT-GTT-GAA-TCT-GGG-TTG-TCT-TCT-
L F P I S W Q M P V E S G L S S

GAG-GAT-TCT-GCA-AGC-TCA-GAA-AGC-TTT-GCT-TCG-AAG-ATT-AAG-CGA-3'
E D S A S S E S F A S K I K R

IgK signal (SEQ ID NO:17 and 18)

5'-ATG-GAG-ACA-GAC-ACA-CTC-CTG-CTG-TGG-GTA-CTG-CTG-CTC-TGG-GTT-CCA-
M E T D T L L L W V L L L W V P

GGT-TCC-ACT-GGT-GAC-3'
G S T G D

Examples

Example 1

GLP-1(7-37)-6xHis Expression Cassette With Igk Signal Sequence

First the GLP-1 sequence along with the signal sequence is designed. Then using the properly designed oligos, a PCR-based gene synthesis method (Withers-Martinez et al., 1999) is used to construct the signal-sequence-GLP-1 (ssGLP-1) construct. For example, the oligos listed in Table 4 are utilized to construct a ssGLP-1 construct containing a murine IgK signal sequence (Seq ID:17 and 18, nucleotide and amino acid sequence, respectively).

Table 4

Ig kappa signal, GLP-1(7-37) with 6xHis-tag - Oligos for Gene Synthesis

	Upper Strand	SEQ ID NO:
Oligo Label	Oligo Sequence	
UG1	5'-ATACAGCTCGAGACGCGTAT-3'	19
UG2	5'-GGAGACAGACACACTCCTGCTGTGGGTACTGCTGCTCTGG-3'	20
UG3	5'-GTTCCAGGTTCCACTGGTGACGCGCACGCTGAAGGGACCT-3'	21
UG4	5'-TTACCAGTGACGTAAGTTCTTATTTGGAAGGCCAAGCTGC-3'	22
UG5	5'-CAAGGAATTCATTGCTTGGCTGGTGAAAGGCCGAGGACAT-3'	23
UG6	5'-CATCATCATCATCATTGATCTAGAGTCGACGTAATCCGCT-3'	24

	Lower Strand	
Oligo Label	Oligo Sequence	
LG1	5'-AGCGGATTACGTCGACTCTA-3'	25
LG2	5'-GATCAATGATGATGATGATGATGTCCTCGGCCTTTCACCA-3'	26
LG3	5'-GCCAAGCAATGAATTCCTTGGCAGCTTGGCCTTCCAAATA-3'	27
LG4	5'-AGAACTTACGTCACTGGTAAAGGTCCCTTCAGCGTGCGCG-3'	28
LG5	5'-TCACCAGTGGAACCTGGAACCCAGAGCAGCAGTACCCACA-3'	29
LG6	5'-GCAGGAGTGTGTCTGTCTCCATACGCGTCTCGAGCTGTAT-3'	30

Gene synthesis is performed with the oligos in Table 4 as described in Withers-Martinez et al., 1999. Oligos were ordered from Life Technologies (Frederick, MD). Briefly, all oligos are diluted to a concentration of 100μM. Equal volumes of each oligo are combined, creating a solution which is further diluted to a concentration of 450nM. The first PCR cycle (gene assembly step) is carried out using Pfu polymerase (Stratagene, La Jolla, California). The gene assembly PCR reaction is performed by running the following reaction.

Reaction Mixture	PCR Cycle
5 ul Oligo Mix (450 nM)	Step#1 94°C - 60 seconds
5 ul 10x Cloned Pfu PCR Buffer (Stratagene)	Step#2 94°C - 30 seconds
1 ul dNTPs (10 mM each)	Step#3 52°C - 30 seconds
38 ul Water	Step#4 72°C - 120 seconds
1 ul Pfu Polymerase	Step#5 Goto step#2 26 times
	Step#6 72°C - 10 Minutes

The gene amplification PCR is performed by running the following PCR reaction.

Reaction Mixture	PCR Cycle		
5 ul Gene assembly reaction	Step#1	94°C -	60 seconds
5 ul 10x Cloned Pfu PCR Buffer (Stratagene)	Step#2	94°C -	45 seconds
1 ul dNTPs (10 mM each)	Step#3	51.4°C -	45 seconds
37 ul Water	Step#4	72°C -	5 minutes
1 ul Pfu Polymerase	Step#5	Goto step#2 25-times	
0.5 ul UG1 primer 100 μM	Step#6	72°C -	10 minutes
0.5 ul LG1 primer 100 μM			

The gene amplification PCR is analyzed by gel electrophoresis and the desired PCR fragment is isolated from the gel using Stratagene's Gel Extraction Kit (Stratagene, La Jolla, California). The isolated fragment is digested with XhoI and XbaI and ligated into pCi (Promega, Madison, WI) that is previously digested with XhoI and XbaI, creating the plasmid pCiGLP1IgK+Ala (Figure 1A). This GLP-1 expression cassette consists of, from 5' to 3', a promoter operably linked to a sequence encoding GLP-1, a signal sequence to allow secretion of GLP-1 from transduced cells, a coding region of GLP-1 with a 6xHistidine tag and a polyadenylation signal. The 6x Histidine tag is utilized for purification of the GLP-1 protein. It is not necessary for expression of GLP-1 or utilization of the invention.

Example 2

GLP-1(7-37)-6xHis Expression Cassette With Exendin Signal Sequence

This construct is cloned using the same procedure as described in Example 1. The procedure is performed with the oligos from Table 5 and the resulting PCR fragment is cloned into pCi (Promega, Madison, WI) as described in Example 1. The resulting plasmid is designated pCiGLP1Ext (Figure1B)

Table 5

Exendin signal, GLP-1(7-37) with 6xHis-tag - Oligos for Gene Synthesis

Upper Strand

Oligo Label	Oligo Sequence	SEQ ID NO:
UG1	5'-ATACAGCTCGAGACGCGTAT-3'	19
UG2-EX	5'-GAAAATCATCCTGTGGCTGTGTGTTTTTGGGCTGTTCCTT-3'	31
UG3-EX	5'-GCAACTTTATTCCCTATCAGCTGGCAAATGCCTGTTGAAT-3'	32
UG4-EX	5'-CTGGGTTGTCTTCTGAGGATTCTGCAAGCTCAGAAAGCTT-3'	33
UG5-EX	5'-TGCTTCGAAGATTAAGCGACACGCTGAAGGGACCT-3'	34
UG4	5'-TTACCAGTGACGTAAGTTCTTATTTGGAAGGCCAAGCTGC-3'	22
UG5	5'-CAAGGAATTCATTGCTTGGCTGGTGAAAGGCCGAGGACAT-3'	23
UG6	5'-CATCATCATCATCATTGATCTAGAGTCGACGTAATCCGCT-3'	24

Lower Strand

Oligo Label	Oligo Sequence	
LG1	5'-AGCGGATTACGTCGACTCTA-3'	25
LG2	5'-GATCAATGATGATGATGATGATGTCCTCGGCCTTTCACCA-3'	26
LG3	5'-GCCAAGCAATGAATTCCTTGGCAGCTTGGCCTTCCAAATA-3'	27
LG4-EX	5'-AGAACTTACGTCACTGGTAAAGGTCCCTTCAGCGT-3'	35
LG5-EX	5'-GTCGCTTAATCTTCGAAGCAAAGCTTTCTGAGCTTGCAGA-3'	36
LG6-EX	5'-ATCCTCAGAAGACAACCCAGATTCAACAGGCATTTGCCAG-3'	37
LG7-EX	5'-CTGATAGGGAATAAAGTTGCAAGGAACAGCCCAAAAACAC-3'	38
LG8-EX	5'-ACAGCCACAGGATGATTTTCATACGCGTCTCGAGCTGTAT-3'	39

Example 3

GLP-1(7-37) Expression Cassette With Igk Signal Sequence and a Propeptide Sequence

The plasmid, pCi-nGLP1IgkplfHis (Figure 1C), contains the CMV promoter, a chimeric intron, the murine Igk signal sequence (isolated from pSecTag2, InVitrogen, Carlsbad, CA), a 30 aa prosequence isolated from the pSecTag2 polylinker, a furin cleavage

site (consensus Arg-Thr-Lys-Arg (SEQ ID:18); Groskreutz et al., 1994), the GLP-1 coding region, a 6-His tag (isolated from pSecTag2), and the Sv40 late polyadenylation signal. This plasmid is constructed using the same procedure as described in Example 1, with the following few exceptions. The gene assembly PCR is performed with the oligos from Table 6. The concentration of Oligos Mix is 348 nM. The gene amplification PCR is performed with a temperature of 62.4°C in step #3. The resulting PCR fragment is cloned into pCi-neo (with an SV40 origin of replication). The resulting plasmid is designated pCi-nGLP1IgkplfHis (Figure 1C).

Table 6

IgK signal, Propeptide sequence, Furin cleavage site, GLP-1(7-37) with 6xHis-tag Oligos for gene synthesis

Upper Strand

Oligo Label	Oligo Sequences	SEQ ID NO:
UG1	5'-ATACAGCTCGAGACGCGTAT-3'	19
UG2	5'-GGAGACAGACACACTCCTGCTGTGGGTACTGCTGCTCTGG-3'	20
UGF3	5'-GTTCCAGGTTCCACTGGTGACGCGGCCCAGCCGGCCAGGCGCGCCG-3'	40
UGF4	5'-TACGAAGCTTGGTACCGAGCTCGGATCCACTCCAGTGTGGTGGAAT-3'	41
UGF5	5'-TCTGCAGATATCCAGCACAAGGACCAAGCGGCACGCTGAAGGGACCT-3'	42
UG4	5'-TTACCAGTGACGTAAGTTCTTATTTGGAAGGCCAAGCTGC-3'	22
UG5	5'-CAAGGAATTCATTGCTTGGCTGGTGAAAGGCCGAGGACAT-3'	23
UG6	5'-CATCATCATCATCATTGATCTAGAGTCGACGTAATCCGCT-3'	24

Lower Strand

Oligo Label	Oligo Sequences	
LG1	5'-AGCGGATTACGTCGACTCTA-3'	25
LG2	5'-GATCAATGATGATGATGATGATGTCCTCGGCCTTTCACCA-3'	26
LG3	5'-GCCAAGCAATGAATTCCTTGGCAGCTTGGCCTTCCAAATA-3'	27
LGF4	5'-AGAACTTACGTCACTGGTAAAGGTCCCTTCAGCGTGCCGCTTG-3'	43
LGF5	5'-GTCCTTGTGCTGGATATCTGCAGAATTCCACCACACTGGAGTGGATC-3'	44
LGF6	5'-CGAGCTCGGTACCAAGCTTCGTACGGCGCGCCTGGCCGGCTGGGCC-3'	45
LGF7	5'-GCGTCACCAGTGGAACCTGGAACCCAGAGCAGCAGTACCCACA-3'	46
LG6	5'-GCAGGAGTGTGTCTGTCTCCATACGCGTCTCGAGCTGTAT-3'	30

Example 4

Analysis of In vitro GLP-1 expression

The GLP-1 plasmid constructs are tested in vitro for GLP-1 expression. For this analysis, 30 ug of the GLP-1 expression plasmids (pCi-nGLP1IgkplfHis, pCiGLP1Ext), are transiently transfected into 293T cells using the $CaPO_4$ transfection method. 48 hrs after

transfection, media is collected and assayed for the presence of GLP-1 by ELISA (Linco Research Inc, St. Charles, Missouri). The standard curve, using purified GLP-1 (7-36 amide) at concentrations ranging from 0-100 pM, and the OD readings are displayed in Table 7 (R^2=0.9919). Using this standard curve, the concentrations of GLP-1 expressed from the plasmid constructs is extrapolated (Table 8). Both GLP-1 expression constructs express measurable amounts of GLP-1. The exendin plasmid (pCiGLP1Ext, designated as exendin in Table 8) expresses the highest levels of GLP-1, approximately 310 pM, while the Igκ plasmid (pCi-nGLP1IgkplfHis, designated as GLP1-1 and GLP1-2 in Table 8) expresses approximately 60 pM.

GLP1(7-36) amide Standards (pM)	ELISA Reading Fluor. Units
0	13.5
2	18.5
5	25.5
10	29.0
20	48.5
50	165.5
100	348.5

Table 7. The OD readings using purified GLP-1 (7-36 amide) peptide in the GLP-1 ELISA (Linco Research Inc.). The standard concentrations ranged from 0-100 pM. R^2=0.9919

Sample	ELISA Reading Fluor. Units	GLP1 Concentration (pM)
GLP1-1+	**227**	**66.12**
GLP1-1-	**138**	**40.43**
GLP1-2+	**184**	**53.71**
GLP1-2-	**299**	**86.9**
Exendin +	**1132**	**327.35**
Exendin -	**1001**	**289.54**
GFP+	**16**	**5.21**
GFP-	**12**	**4.06**

Table 8. GLP-1 ELISA results using supernatant collected from 293T cells transfected with the GLP-1 expression cassettes. The GLP-1 concentrations were calculated from the standard curve (Table 7). GLP1-1, and GLP1-2, designate media isolated from cells transfected with pCi-nGLP1IgkplfHis. The 1 and 2 designations indicate two different clones

of the pCi-nGLP1IgkplfHis plasmid. Exendin designates media isolated from cells transfected with (pCiGLP1Ext). The (+) or (-) indicates the inclusion, or absence of serum in the media, respectively.

These constructs contain a 6-His tag, which is added to aid in protein purification for subsequent NH_2-terminal sequence analysis. It is possible that this tag interferes with the GLP-1 ELISA. To directly test whether this affects the accuracy of the the GLP-1 expression data, various GLP-1 peptides are synthesized, including GLP-1(7-37) with a 6xHis tag, GLP-1 (7-36) amide, GLP-1 (7-36, Ser 8 mutation) amide, GLP-1 (7-36) without amide, and GLP-1 (7-37). Interestingly, the GLP-1 (7-36) amide, and GLP-1 (7-36) without amide show predicted quantitation using the Linco ELISA (Table 9). However, GLP-1 (7-37) GLP-1(7-37) with a 6xHis tag, and GLP-1 (7-36, Ser 8 mutation), show less efficient detection (Table 9). These data suggest that the GLP-1 peptide present in the conditioned media may actually be more concentrated than indicated by the ELISA data.

Peptide	Concentration	ELISA Reading extrapolated concentration (pM)
GLP1(7-36) Amide	10nM	1117
	1nM	109
	100pM	15
	10pM	9
Ser8-GLP1(7-36) Amide	10nM	302
	1nM	17
	100pM	11
	10pM	11
GLP1(7-36) Without amide	10nM	1143
	1nM	117
	100pM	14
	10pM	10
GLP1(7-37)	10nM	734
	1nM	237
	100pM	22
	10pM	21
GLP1(7-37) 6xHIS	10nM	449
	1nM	13
	100pM	19
	10pM	16

Table 9. GLP-1 ELISA results using various synthesized peptides. The type of GLP-1 peptide is displayed in the first column, the actual peptide concentration is displayed in the middle column, and the calculated value is displayed in the last column.

Example 5

The use of an inducible gene expression system will allow the regulation of GLP-1 and GIP in a reversible manner. Several inducible systems are currently available. For example, the Tet-On ™ and Tet-Off ™ systems currently available from Clontech (Palo Alto, CA) allow the regulated expression of the transgene controlled by tetracycline or tetracycline derivatives, such as doxycycline. The Tet-Off system uses the tetracycline-controlled transactivator (tTA), which is composed of the tet repressor protein (TetR) and the VP16 activation domain. tTA activates transcription in the absence of tetracycline. The Tet-On system uses the reverse tetracycline-controlled transactivator (rtTA) and activates transcription in the presence of tetracycline. Both systems use the tetracycline-response element (TRE) which contains 7 repeats of the tet operator sequence, and the target gene, such as GLP-1 or GIP. tTA or rtTA bind to the TRE, activating transcription of the target gene.

To generate tet-controlled GLP-1 and GIP expression cassettes, the expression cassettes described above are cloned into the pTRE2 plasmid (Clontech, Palo Alto, CA) into the multiple cloning sequence. These plasmids, pTRE2-GLP-1 and pTRE2-GIP, in addition to the expression cassette containing the Tet-On or Tet-Off expression elements (contained in plasmid pTet-On or pTet-Off, Clontech), are inserted into the gutless vector plasmid, pGTI14 or pGTI15, and a gutless vector encoding both expression cassettes is generated (Figure 2) by methods known to those skilled in the art.

Example 6

Generation and production of gutless adenoviral vectors encoding GLP-1 and/or GIP expression cassettes

The GLP-1 and GIP-1 gutless adenoviral vectors are generated in the following manner. To make the gutless vector plasmids, bp 1-384 of Ad5, which contains the ITR and viral packaging signal is PCR amplified using the following oligonucleotides:

#1 (5'- GCAGGTACCTTAATTAACATCATCAATAATATACCTTATTTTG 3') (SEQ ID NO:47)

#2 (5'- GAACTGCAGGTCTCCACGTAAACGGTCAAAG 3') (SEQ ID NO:48)

The adenoviral sequences are displayed in bold. Pac I and Pst I restriction enzyme sites are engineered in oligonucleotides 1 and 2, respectively. Next, 420 bp of the right end of Ad5 (bp 35,516 to 35,935) is PCR amplified using oligonucleotide #3 (5' GAACTGCAGGGCAGCCTAACAGTCAGCCTTACC 3') (SEQ ID NO:49), encoding a Pst I site, and oligonucleotide #1 (above). The PCR products are digested with Pst I and inserted into pBluescript II (Stratagene, La Jolla, CA) digested with Ssp I and Pvu II in a three-way ligation, to construct pGTL10a. Therefore, pGTI10a contains both termini of the Ad5 genome bordered by Pac I sites. In the next step, pGTL11a is generated from pGTL10a by the removal of 125 bp from the right terminal fragment including the cap site and TATA box from the promoter region of E4, Ad5 bps 35,516 to 35,640. Next, pGTL14 and pGTL15 are generated from pGTL10a and pGTL11a, respectively, following insertion of 21.9 kb human synuclein intronic region stuffer sequence. The final gutless vector plasmids are generated by insertion of the GLP-1 and/or the GIP expression cassettes into pGTL14 and/or pGTL15. A 4 kb DNA stuffer sequence, derived from human genomic DNA C346 (Sandig et al., 2000), which is isolated from human cells by PCR amplification is inserted into pGTL14 and pGTL15 to generated pGTL14C346 and pGTL15C346, respectively. The final gutless vector plasmids are generated by insertion of one or several GLP-1 and/or the GIP expression cassettes into pGTL14C346 and/or pGTL15C346. Examples of these resulting plasmids are pGTL14C346GLP-1 and pGTL15C346GLP-1, respectively (Figures 3).

The helper virus, Av1nBgflx, encodes a β-galactosidase (β-gal) marker gene in a viral backbone lacking E1 and E3. Av1nBgflx is generated by cotransfecting two plasmids, pAV6alxirsvnbgspa and pSQ1 into PER.C6 cells (Fallaux et al., 1998) following standard protocols. The plasmid, pAV6alxirsvnbgspa is constructed in several steps. First, pAvS6 (Gorziglia et al., 1996), encoding Ad5 bp 1 to 393 and 6246 to 3509 is modified by insertion of a 34 bp long loxP site at Ad5 bp 191, to produce pAV6alxa. Next, a second loxP site is inserted into pAV6alxa at Ad5 bp 393, to generate pAV6alxi. In the final step, the β-gal expression cassette, encoding an RSV promoter, nuclear localized β-gal, and a synthetic poly(A) signal, is inserted into pAV6alxi to generate pAV6alxirsvnbgspa. The plasmid pSQ1 contains the majority of the Ad5 genome excluding the E1 and E3 regions, Ad5 bp position

3,329 to 28,591 and 30,470 to 35,935. Viral genome integrity is confirmed by restriction analysis of DNA isolated from CsCl-purified vector.

Gutless vector generation from plasmid, and large scale vector production is performed using C7-Cre cells (Hartigan-O'Connor, et al., 1999), which are a 293-based cell line, further modified to express the Ad5 E2b proteins (pTP and Pol) and Cre recombinase of P1 bacteriophage. Gutless vector sequences are liberated from pGTI.14GLP-1 and pGTI.15GLP-1 by Pac I digestion and transfected into sub-confluent monolayers of C7-Cre cells in a six well dish using the Profection calcium phosphate transfection method (Promega, Madison, WI). Twelve to sixteen hours after transfection, the cells are infected with helper virus at a dose of 100 particles/cell. Cells are harvested when complete cytopathic effect (CPE) is observed. Cells are subjected to three cycles of freeze-thaw, and the lysate is used to infect one 150 mm dish of C7-Cre cells with helper virus supplementation, 100 particles/cell. When CPE is complete (approximately 48 hrs after helper virus addition), the cell lysate is used to infect 10 x 150-mm plates of C7-Cre cells with helper virus supplementation. Gutless vectors are purified by CsCl gradient centrifugation. CsCl purified gutless vector preparations are subsequently used as seedlots for vector production. Using the gutless vector seed lot, C7-Cre cells are infected with 30 particles of gutless vector/cell and with helper virus at 100 particles /cell.

To confirm the correct vector is isolated, DNA from purified gutless vectors is extracted and digested with BamHI and the ends of the resulting DNA fragments are labelled with α-^{33}P-dGTP using Klenow fragment. The restriction enzyme fragments are then resolved on agarose gel stained with ethidium bromide and photographed. Next, the DNA fragments are transferred from agarose gel to Hybond-N and subjected to autoradiography. The bands from the gel are observed to see if they match the expected sizes for the correct gutless vector.

Hexon Taqman real-time PCR assay: PCR primers and a Taqman probe specific to adenovirus hexon sequences are designed using Primer Express software V. 1.0 (Applied Biosystems, Foster City, CA). Primer and probe sequences are:

hexon forward primer: 5' CTTCGATGATGCCGCAGTG-3' (SEQ ID NO:50);

hexon reverse primer: 5' GGGCTCAGG TACTCCGAGG-3' (SEQ ID NO:51);

hexon probe: 5' FAM-TTACATGCACATCTCGGGCCAGGAC-TMRA-3' (SEQ ID NO:52).

Amplification is performed in a reaction volume of 50 ul under the following conditions: 1,500,000 copies of vector DNA, 1X Taqman universal PCR master mix (Applied Biosystems), 600 nM forward primer, 900 nM reverse primer and 100 nM hexon probe. Thermal cycling conditions are: 2 minutes incubation at 50^0C, 10 minutes at 95^0C, followed by 35 cycles of successive incubation at 95^0C for 15 seconds and 600C for 1 minute. Data is collected and analysed using 7700 Sequence Detection System software v.1.6.3. (Applied Biosystems). Quantification of adenovirus copy number is performed using standard curve consisting of dilutions of gutless adenovirus DNA from 1,500,000 copies to 15 copies. The percent helper virus is calculated as the average hexon copy number from three PCR replicates divided by 1,500,000 total vector copies.

For quantitation of the β-gal-encoding helper virus, 1.75×10^5 AE1-2a cells (Gorziglia et al., 1996) are seeded in 24-well plates. The cells are infected with multiplicities of infections of 2000, 1000, 500, 250 and 125 viral particles/cell. Twenty-four hours following infection, the cells are fixed and stained with X-gal (Smith et al., 1993) and beta-gal positive cells are counted.

The vector preparations are checked for the presence of replication-competent adenovirus (RCA) contamination by PCR directed at E1a sequences (Tolstoshev et al., 1994). All vector concentrations are determined by spectrophotometric analysis (Mittereder et al., 1996). Titers are given as viral particles per milliliter.

The gutless vectors described in Examples 6A-6C are generated as described above in Example 6.

Example 6A

Generation of AGV-C7-GLP

The gutless adenoviral vector, AGV-C7-GLP contains a GLP-1 expression cassette composed of the exendin signal sequence, GLP-1 coding region, and SV40 early polyadenylation signal, driven the by the inducible regulated promoter. This promoter has been described by Xu et al. (2001). The plasmid, pBV2.CGE.C7.GLP-1Ex (Figure 4A), used to generate the gutless vector AGV-C7-GLP, is constructed in the following manner. First, the C7, inducible promoter region is isolated from pAGVC7endo1c (Figure 4B) using NheI and Pme I. The exendin/GLP-1 coding region is isolated from pCiGLP1Ex (described in Example 2; Fig. 1B), using Nhe I and Cla I. Both fragments are combined with pGTI.24aPL2 (Figure 4C) to generate pGTI.APL2.CGE.C7.GLP-1Ex (Figure 4D). Then,

pGTLAPL2.CGE.C7.GLP-1Ex is digested with PacI to liberate the plasmid backbone and combined with PmeI and MluI digested pBV2 (Figure 4E). The final gutless vector is generated by homologous recombination in BJ5138 E.coli as described by Toietta et al. (2002).

The gutless vector, AGV-C7-GLP is generated following transfection of the plasmid into C7-Cre cells (Reddy et al., 2002) which are a 293-based cell line, further modified to express the Ad5 E2b proteins (pTP and Pol) and Cre recombinase of P1 bacteriophage. Gutless vector sequences are liberated from pBV2.CGE.C7.GLP-1Ex by digestion with Pac I and 50 ug of DNA per plate is transfected into sub-confluent monolayers of C7-Cre cells in 3-x15 cm tissue culture plates using the Profection calcium phosphate transfection method (Promega, Madison, WI). Twelve to sixteen hours after transfection, the cells are infected with the helper virus, Av1S4BflxFE3 (Sakhuja et al., 2002) (in press) at a dose of 100 particles/cell. Cells are harvested when complete cytopathic effect (CPE) is observed. Cells are subjected to three cycles of freeze-thaw, and the lysate is used to infect 10 x 15 cm plates of C7-Cre cells with helper virus supplementation. Gutless vectors are purified by CsCl gradient centrifugation. CsCl purified gutless vector preparations are subsequently used as seedlots for vector production. Using the gutless vector seed lot, C7-Cre cells are infected with 30 particles of gutless vector/cell with helper virus at 100 particles /cell. A CsCl gradient is used to purify the AGV-C7-GLP gutless vector. A total yield of 2×10^{12} particles in the 30x15 cm plates was obtained. DNA from this purified gutless vector is extracted and digested with Not I and restriction enzyme fragments are resolved on an agarose gel stained with ethidium bromide. The expected restriction enzyme pattern after Not I digestion for the gutless vector is: 19515, 5003, 3217, 415, and 42, and for the helper virus: 12193, 7932, 6428, 5001, 2589, 1931, 960, and 326. The identity and integrity of the gutless vector genome are verified by restriction digest.

Example 6B

Generation of AGV-CMV-GLP

The gutless adenoviral vector, AGV-CMV-GLP contains a GLP-1 expression cassette composed of the exendin signal sequence, GLP-1 coding region, and SV40 early polyadenylation signal, driven by the CMV promoter. The plasmid, pBV2.CMV.GLP-1Ex (Figure 5A), used to generate the gutless vector AGV-CMV-GLP, is constructed in the following manner. First, the plasmid, pGTLAPL2.CMV.GLP-1Ex (Figure 5B) is constructed

by digesting pCiGLP1Ex with Bgl II, filling in the ends, and digesting with ClaI and ligating to pGTL24APL2 digested with SmaI and NarI. Then pGTLAPL2.CMV.GLP-1Ex, is digested with Pac I to liberate the plasmid backbone and combined with PmeI and MluI digested pBV2 (Figure 4E). The final gutless vector is generated by homologous recombination in BJ5138 *E.coli* as described by Toietta *et al.*, (2002).

The AGV-CMV-GLP gutless vector is generated and propagated as described above. Supernatant collected from cells propagating the AGV-CMV-GLP gutless vector, collected from the 10-plate CVL stage, is assessed for GLP-1 expression by ELISA (Linco Research Inc., St. Charles, Missouri), as described. The data are displayed in Table 10.

Sample	ELISA Reading	GLP-1 (pM)
GLP-1 Standards (pM)		
0	27	
2	54	
5	84	
10	127	
20	305	
50	942	
100	1805	
Quality Controls		
#1 (Expected range 4-9 pM)	87	5.3
#2 Expected range 44-92 pM)	1200	66.2
Samples : CMV –GPLP-1 AVG		
#1	73	4.22
#2	57	3.67
#3	47	3.1

Table 10. GLP-1 ELISA results from CVL of C7 cre cells infected with AGV-CMV-GLP. The CVL was collected from one 150mm plate 48hrs following the initial stage of virus production.200ul of CVL was used for each well.Data represents the mean values of three wells. GLP-I concentrations were calculated from a standard curve.

Example 6C
Generation of AGV-ALB-GLP

The gutless adenoviral vector, AGV-ALB-GLP contains a GLP-1 expression cassette composed of the exendin signal sequence, a liver-specific albumin promoter operably linked to a GLP-1 coding region, and SV40 early polyadenylation signal. First, the albumin promoter and heterologous intron from the Apo A1 gene, is isolated by PCR amplification of the plasmid, pAvALAPH81 (Connelly et al., 1996). The oligonucleotide primers for the PCR amplification are as follows: 5' GATATGTTTAAACACGCGTGCTATGACC 3' (SEQ ID NO:53) with the addition of a Pme I site (bold underline), and 5' CCTGGGCTAGCAGGAGAAGAAGGGC 3' (SEQ ID NO:54) with the addition of an NheI site (bold underline). The PCR fragment is digested with PmeI and NheI and combined with NheI and ClaI digested pCiGLP1Ex and inserted into pGTL24ALP2 digested with PmeI and NarI to obtain pGTLAPL2.ALB.GLP-1EX (Figure 6A). Next, pGTLAPL2.ALB.GLP-1EX is digested with PacI to liberate the plasmid backbone and combined with PmeI and MluI digested pBV2, to create pBV2.ALB.GLP-1EX (Figure 6B). The final gutless vector is generated by homologous recombination in BJ5138 *E.coli* as described by Toietta *et al.*, (2002).

Example 7

Generation and production of BIV vectors encoding GLP-1 and/or GIP expression cassettes

BIV vectors capable of expressing GLP-1 and/or GIP are constructed as described in WO 01/44458, which is incorporated herein by reference in its entirety. Briefly, the expression cassettes as described above are cloned into a BIV vector plasmid. This plasmid is then co-transfected into a BIV producer cell line along with a plasmid expressing a viral envelope protein (e.g. VSVG envelope) and a BIV packaging construct or constructs (WO 01/44458). The supernatant is then harvested from the producer cells. This supernatant contains the GLP-1 encoding BIV vector, which is then used to transduce the desired cell types. Preferably, the BIV vector supernatant is concentrated after harvest.

Example 8

Generation and production of AAV vectors encoding

GLP-1 and/or GIP expression cassettes

AAV vectors encoding GLP-1 and/or GIP can be generated following standard protocols known to those skilled in the art. Briefly, AAV vectors are constructed by the co-transfection method where a plasmid encoding the AAV vector, composed of the AAV inverted terminal repeat (ITR) sequences and the GLP-1 or GIP expression cassette is cotransfected with an AAV helper plasmid as described previously (Xiao et al., 1996). Subconfluent 293 cells are cotransfected with the AAV plasmid and helper plasmid using calcium phosphate precipitation. Forty-eight hours after transfection, the cells are harvested, lysed by 3 cycles of freeze-thawing and sonicated to release the AAV vector. Following ammonium sulfate precipitation, the virus particles are concentrated twice by cesium chloride density gradient centrifugation.

Example 9

In vivo evaluation of a GLP-1 encoding Gutless adenoviral vector

The GLP-1 encoding gutless adenoviral vectors, AGV-C7-GLP, AGV-CMV-GLP, and AGV-ALB-GLP are evaluated in diabetic mice following systemic vector delivery. This example describes the regulated, AGV-C7-GLP vector, but other vectors of the invention are evaluated in a similar manner.

The ability to regulate GLP-1 expression in a diabetic animal model and in humans is desired to potentially improve the utility of the therapy. The ligand-inducible controlled gene expression (CGE) system has been described (PCT/US02/16946). As our animal model, C57BL/6 mice are fed a high fat diet. These animals become obese and diabetic and display elevated fasting blood glucose levels, elevated insulin levels, and impaired glucose tolerance (Desai et al., 2001). The diabetic mice are treated with 6×10^{10} particles of the AGV-C7-GLP vector and an equal amount (6×10^{10} particles) of a gutless vector encoding a novel transcription factor via tail vein injection. This system is similar to that described in Xu, et al. (2002), except Xu, et al. was expressing endostatin and here, GLP-1 is being expressed. This dose of vector results in efficient transduction of the liver with both vectors (PCT/US02/16946). The small molecule inducing agent, such as tamoxifen, or the tamoxifen derivatives described previously (PCT/US02/16946) are delivered at a dose of 50 ug/mouse

through IP injection, daily, dependent on the length of time and level of GLP-1 expression desired. Animals are monitored for GLP-1 expression in the serum by ELISA (Linco Research Inc., St. Charles, Missouri), and for fasting blood glucose levels, insulin levels, and glucose tolerance. Other parameters are also assessed, including lactate, free fatty acids, triglycerides, and liver enzyme levels. The amount of inducing agent can be varied in response to any of these measured parameters.

This example describes an adenovirus encoding an inducible GLP-1 expression cassette and includes another co-administered gutless adenoviral vector which encodes the necessary transcription factor. In a preferred embodiment, both the inducible GLP-1 expression cassette and the necessary transcription factor are encoded on one gutless adenoviral vector.

Evaluation of the AGV-CMV-GLP and the AGV-ALB-GLP vectors is performed in a similar manner. However, 6×10^{10} particles of the relevant vector are delivered via tail vein injection, and no transcription factor vector or inducer is utilized. The same parameters as described above are evaluated.

The disclosures of all patents, publications (including published patent applications), and database accession numbers referred to in this specification are specifically incorporated herein by reference in their entirety to the same extent as if each such individual patent, publication, and database accession number were specifically and individually indicated to be incorporated in its entirety.

It is to be understood, however, that the scope of the present invention is not to be limited to the specific embodiments described above. The invention may be practiced other than as particularly described and still be within the scope of the accompanying claims.

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What Is Claimed Is:

3. A viral vector comprising:

(a) a polynucleotide sequence encoding GLP1;

(b) a polynucleotide sequence encoding a signal sequence upstream of (a); and

(c) a polyadenylation signal downstream of (a).

4. The vector according to claim 1, further comprising a polynucleotide sequence encoding a proteolytic cleavage site located between said polynucleotide sequence encoding GLP1 and said polynucleotide sequence encoding the signal sequence.

3. The vector according to claim 1 or 2, wherein GLP1 comprises SEQ ID NO:2, 4, 6, 8, or 10.

4. The vector according to claim 1 or 2, wherein said polynucleotide sequence encoding GLP1 comprises SEQ ID NO:1, 3, 5, 7 or 9.

5. The vector according to any of claims 1-4, wherein said signal sequence is an IgK signal sequence.

6. The vector according to claim 5, wherein said IgK signal sequence comprises SEQ ID NO:18.

7. The vector according to claim 6, wherein said polynucleotide encoding the IgK signal sequence comprises SEQ ID NO:17.

8. The vector according to any of claims 1-4, wherein said signal sequence is an exendin signal sequence.

9. The vector according to claim 8 wherein said exendin signal sequence comprises SEQ ID NO:16.

10. The vector according to claim 9, wherein said polynucleotide encoding the exendin signal sequence comprises SEQ ID NO:15.

11. The vector according to any one of the preceding claims, wherein the polyadenylation signal is derived from SV40.

12. The vector according to any one of claims 2-11, wherein said proteolytic cleavage site is cleaved by furin protease.

13. The vector according to any one of the preceding claims, wherein expression of said polynucleotide sequence encoding GLP1 is controlled by a regulatable promoter.

14. The vector according to any of claims 1-13, wherein said vector is an adenoviral vector.

15. The vector according to any of claims 1-13, wherein said vector is a retroviral vector.

16. The vector according to any of claims 1-13, wherein said vector is a lentiviral vector.

17. The vector according to any of claims 1-13, wherein said vector is an adeno associated viral vector.

18. The vector according to any one of the preceding claims, further comprising a polynucleotide encoding GIP.

19. The vector according to claim 18, wherein GIP comprises SEQ ID NO:14.

20. The vector according to claim 19, wherein said polynucleotide sequence encoding GIP comprises SEQ ID NO:13.

21. A mammalian cell comprising the vector according to any one of claims 1-20.

22. A packaging cell comprising the vector according to any one of claims 1-20.

23. A method of making a viral particle comprising culturing the packaging cell of claim 22 under conditions wherein the particle is produced.

24. A method of treating diabetes in a mammal comprising administering a physiologically effective amount of the vector in any one of claims 1-20 to the mammal.

25. The method of claim 24, wherein the mammal is a primate.

26. The method of claim 25, wherein the primate is a human.

27. The method of treating diabetes according to any one of claims 24-26, further comprising administering a DPP-IV inhibitor to said mammal.

28. A viral vector comprising:

(a) a polynucleotide sequence encoding GIP;

(b) a polynucleotide sequence encoding a signal sequence upstream of (a); and

(c) a polyadenylation signal downstream of (a).

29. The vector according to claim 27, further comprising a polynucleotide sequence encoding a proteolytic cleavage site located between said polynucleotide sequence encoding GIP and said polynucleotide sequence encoding the signal sequence.

30. The vector according to claim 28 or 29, wherein GIP comprises SEQ ID NO:14.

31. The vector according to claim 30, wherein said polynucleotide sequence encoding GIP comprises SEQ ID NO:13.

32. The vector according to any of claims 28-31, wherein said signal sequence is an IgK signal sequence.

33. The vector according to claim 32, wherein said IgK signal sequence comprises SEQ ID NO:18.

34. The vector according to claim 33, wherein said polynucleotide encoding the IgK signal sequence comprises SEQ ID NO:17.

35. The vector according to any of claims 28-31, wherein said signal sequence is an exendin signal sequence.

36. The vector according to claim 35, wherein said exendin signal sequence comprises SEQ ID NO:16.

37. The vector according to claim 36, wherein said polynucleotide encoding the exendin signal sequence comprises SEQ ID NO:15.

38. The vector according to any one of claims 28-37, wherein the polyadenylation signal is derived from SV40.

39. The vector according to any one of claims 29-38, wherein said proteolytic cleavage site is cleaved by furin protease.

40. The vector according to any one of claims 28-39, wherein expression of said polynucleotide sequence encoding GIP is controlled by a regulatable promoter.

41. The vector according to any of claims 28-39, wherein said vector is an adenoviral vector.

42. The vector according to any of claims 28-39, wherein said vector is a retroviral vector.

43. The vector according to any of claims 28-39, wherein said vector is a lentiviral vector.

44. The vector according to any of claims 28-39, wherein said vector is an adeno associated viral vector.

45. The vector according to any one of claims 28-44, further comprising a polynucleotide sequence encoding GLP1.

46. A mammalian cell comprising the vector according to any one of claims 28-45.

47. A packaging cell comprising the vector according to any one of claims 28-45.

48. A method of making a viral particle, comprising culturing the packaging cell according to claim 47 under conditions wherein the particle is produced.

49. A method of treating diabetes in a mammal, comprising administering a physiologically effective amount of the vector according to any one of claims 28-45 to the mammal.

50. The method of claim 49, wherein the mammal is a primate.

51. The method of claim 50, wherein the primate is a human.

52. The method of treatment as specified in any one of claim 49-51, further comprising administering a DPP-IV inhibitor to said mammal.

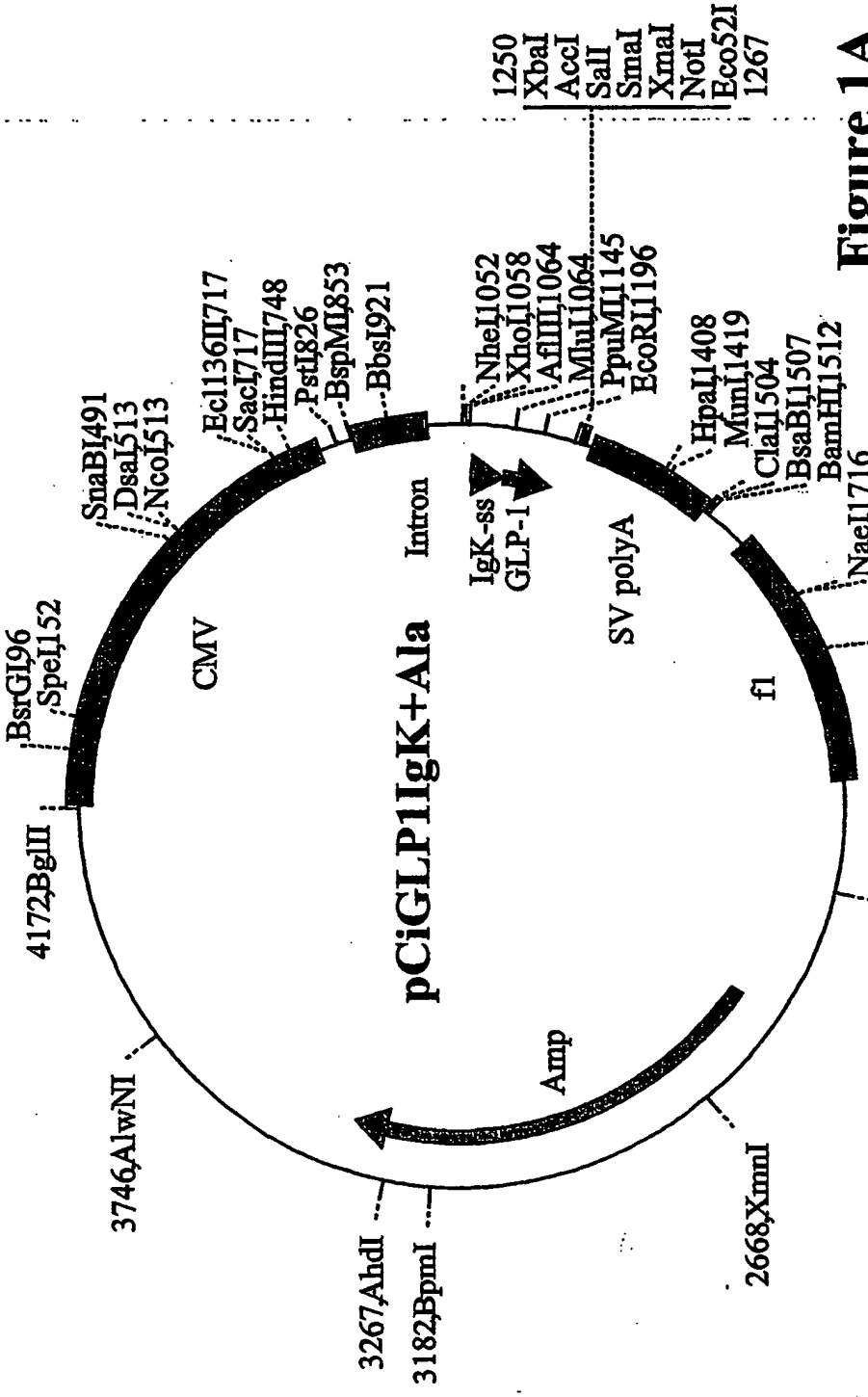


Figure 1A

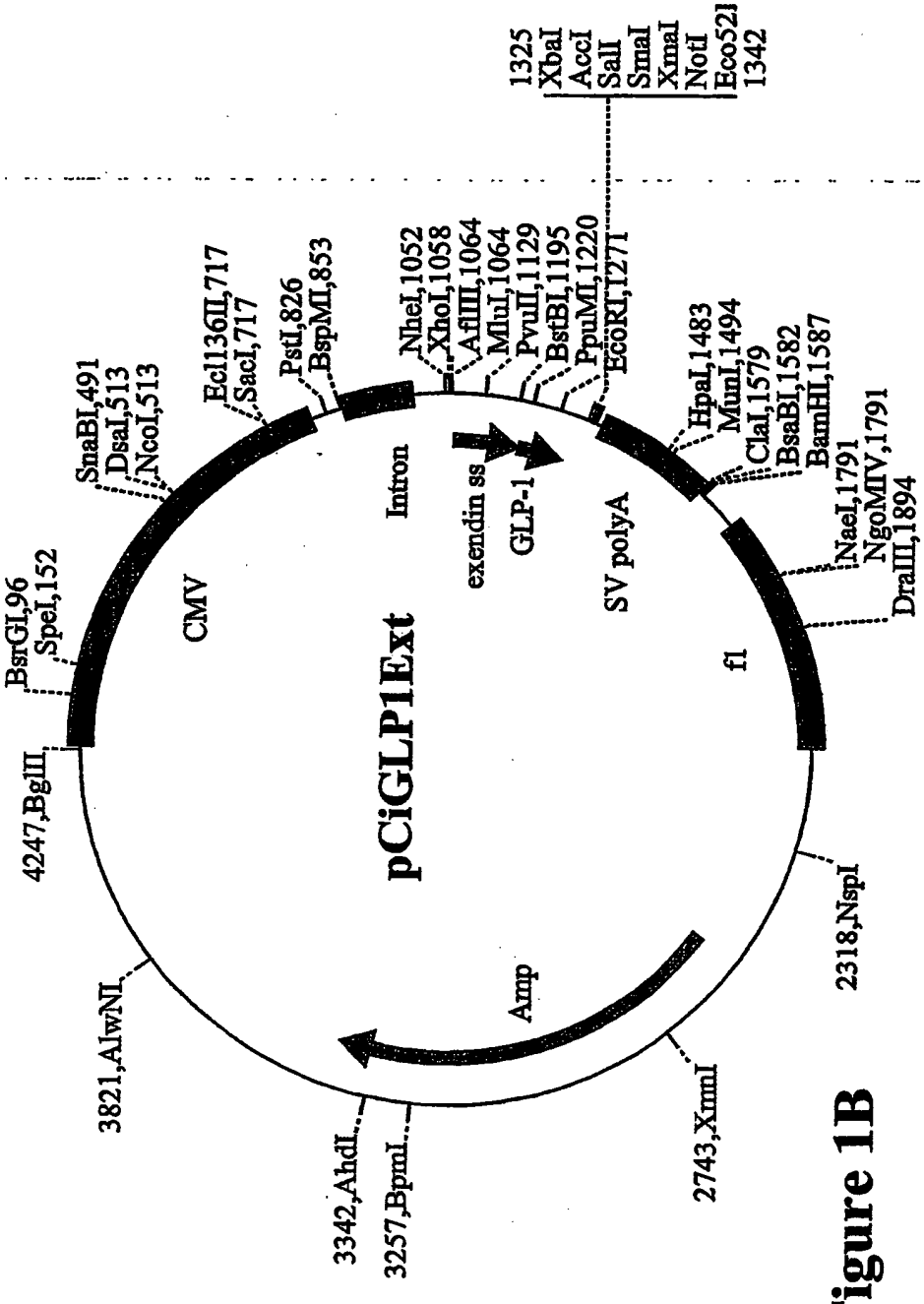


Figure 1B

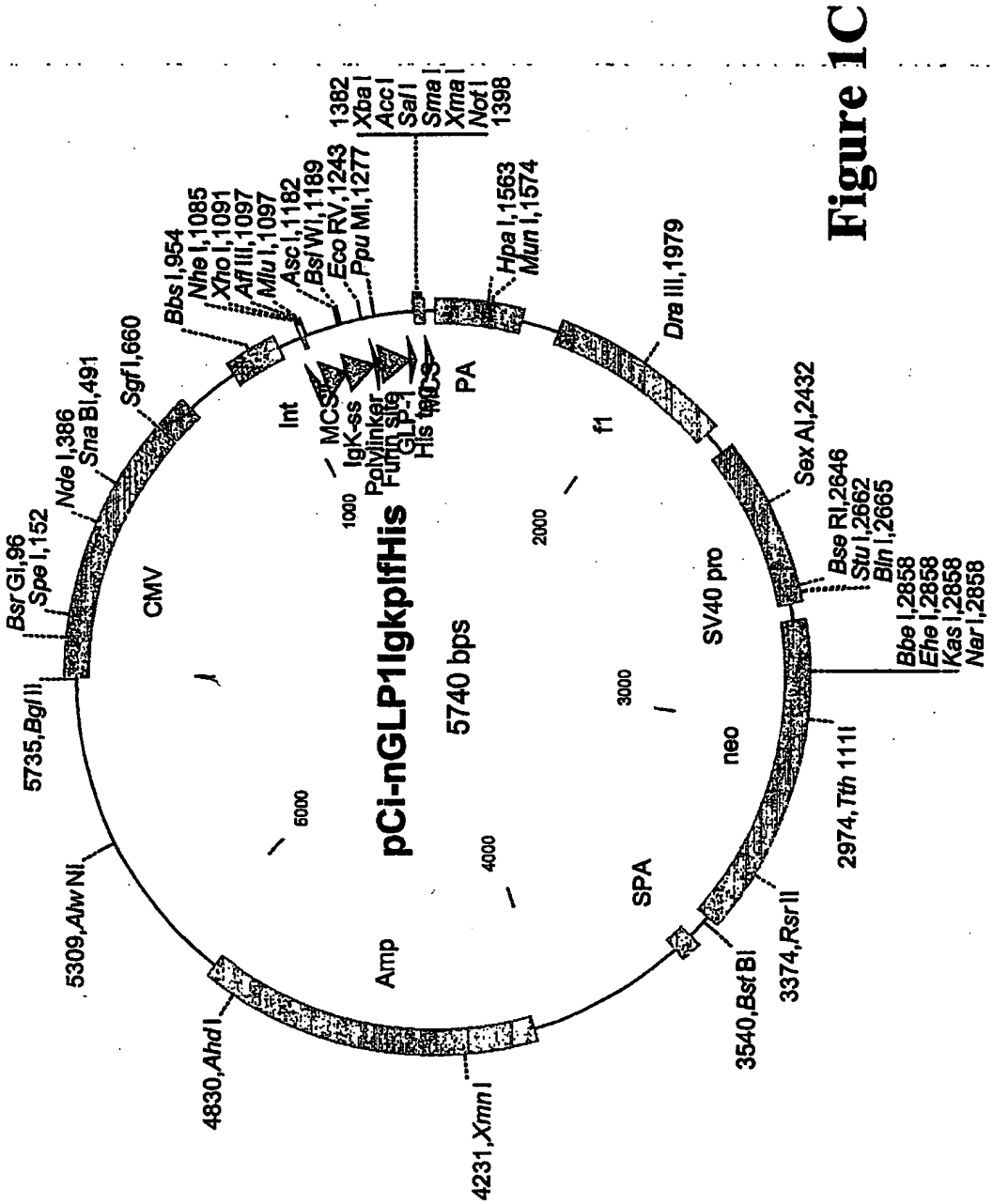


Figure 1C

Adenoviral Vectors EncodingTet System

Gutless Adenoviral Vector encoding Tet-On Regulator

Gutless Adenoviral Vector encoding Tet-Off Regulator

Tetracycline-controlled transactivator (tTA), Tet off (active - tet)

Reverse tetracycline-controlled transactivator (rtTA), Tet on (active + tet)

Tetracycline-response element (TER)

Figure 2

Figure 3A

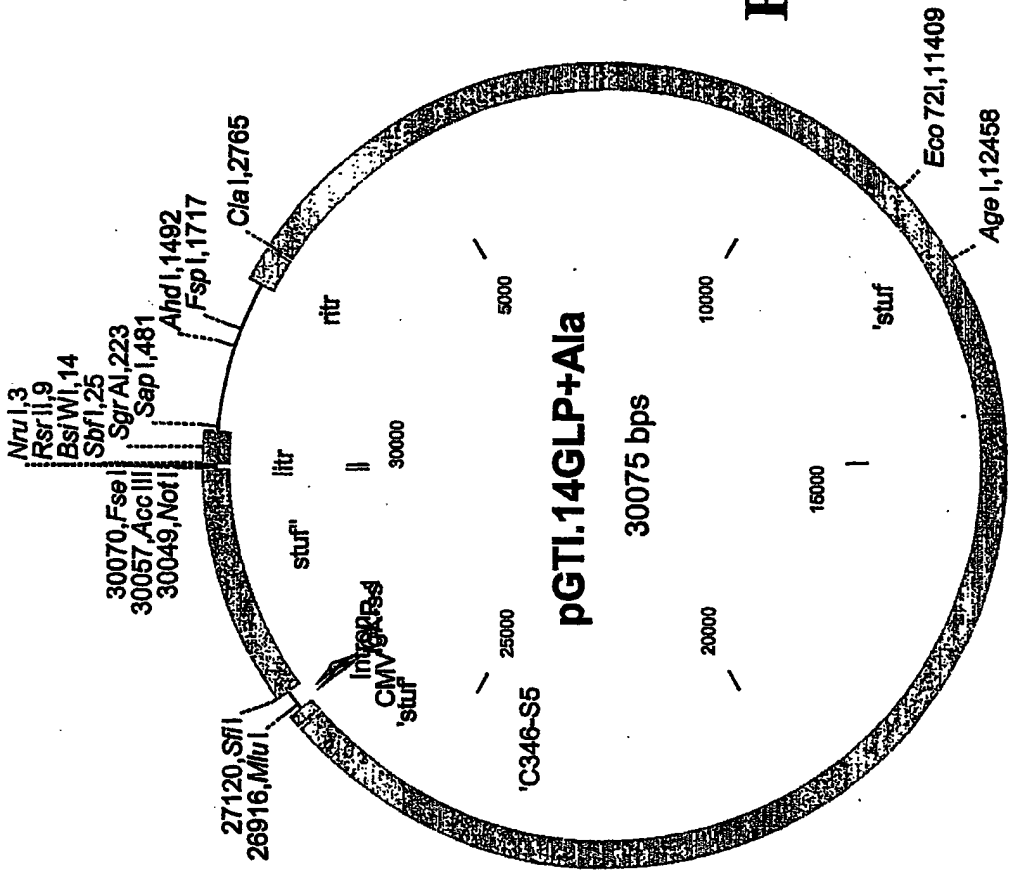
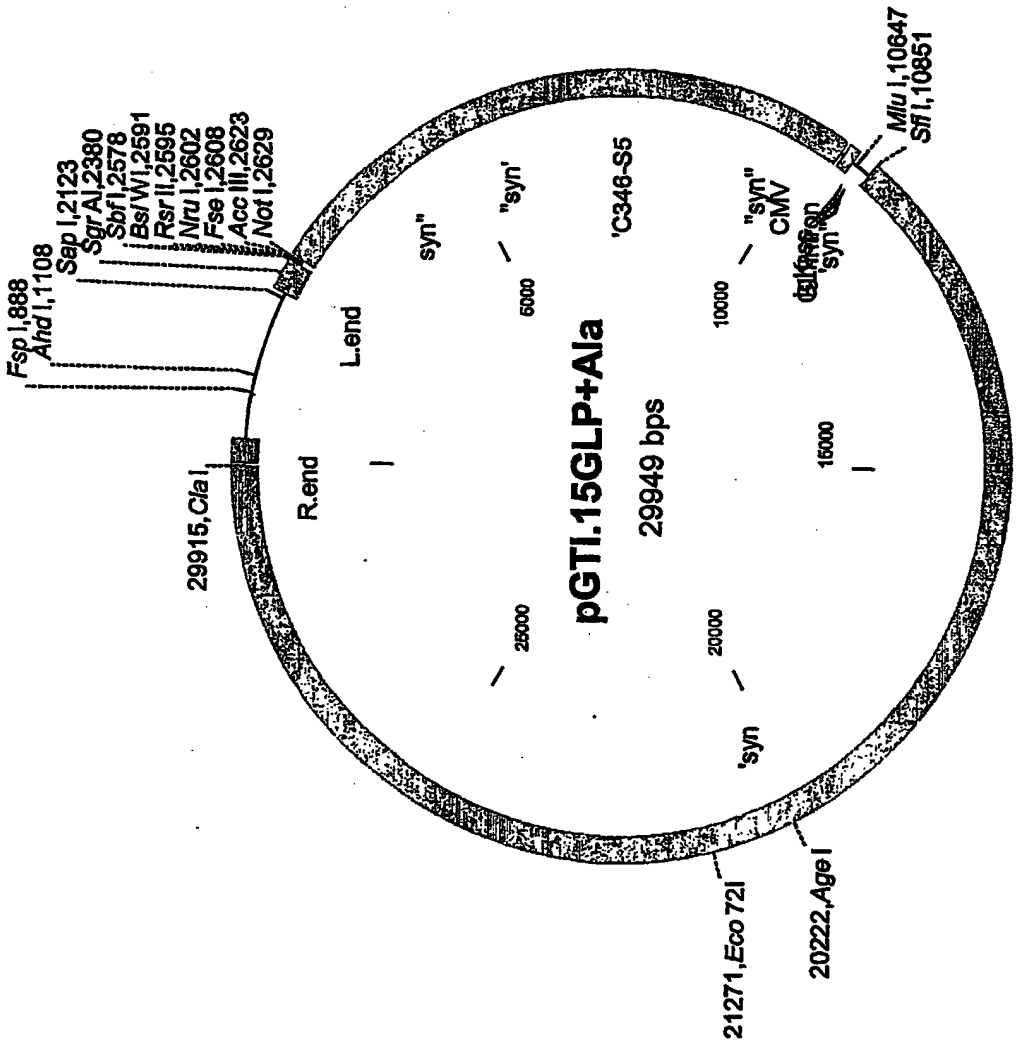


Figure 3B



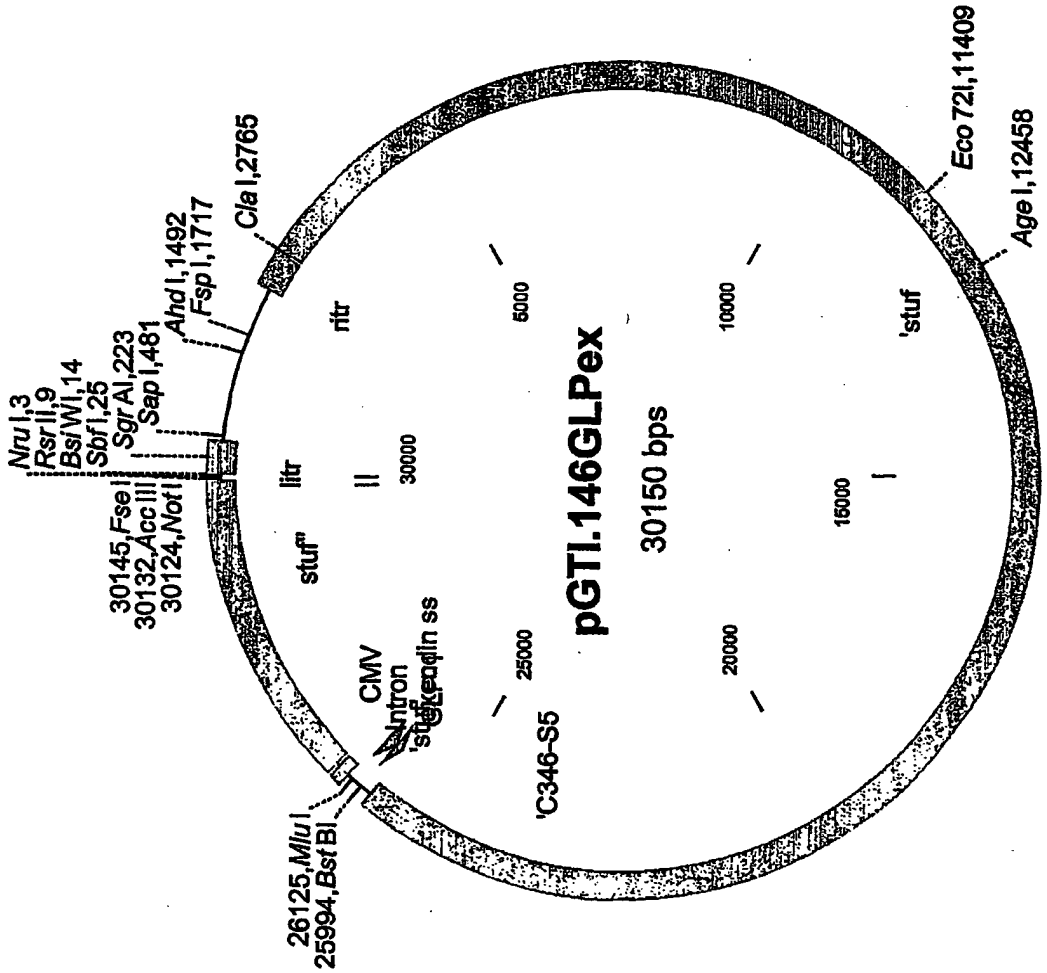


Figure 3C

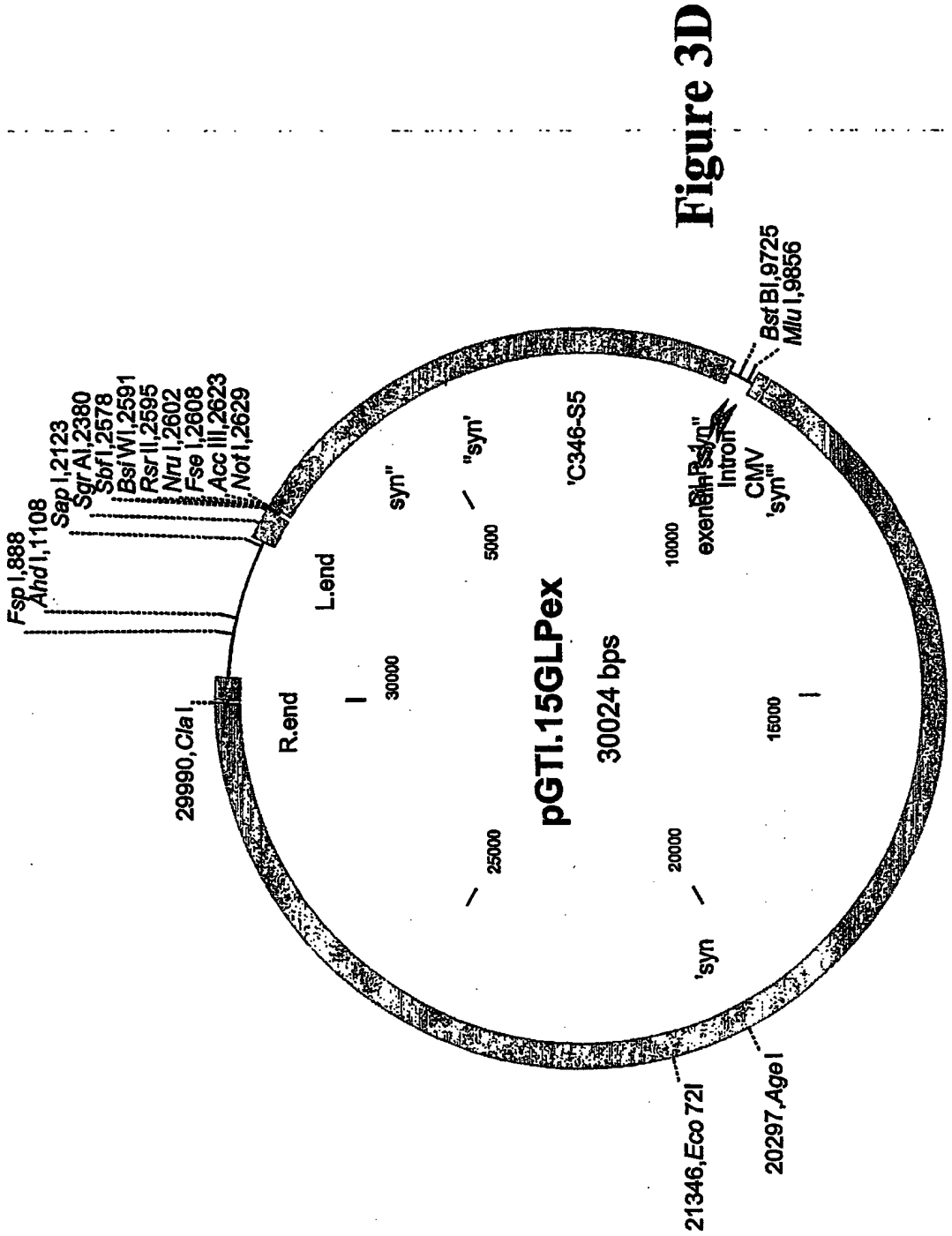


Figure 3D

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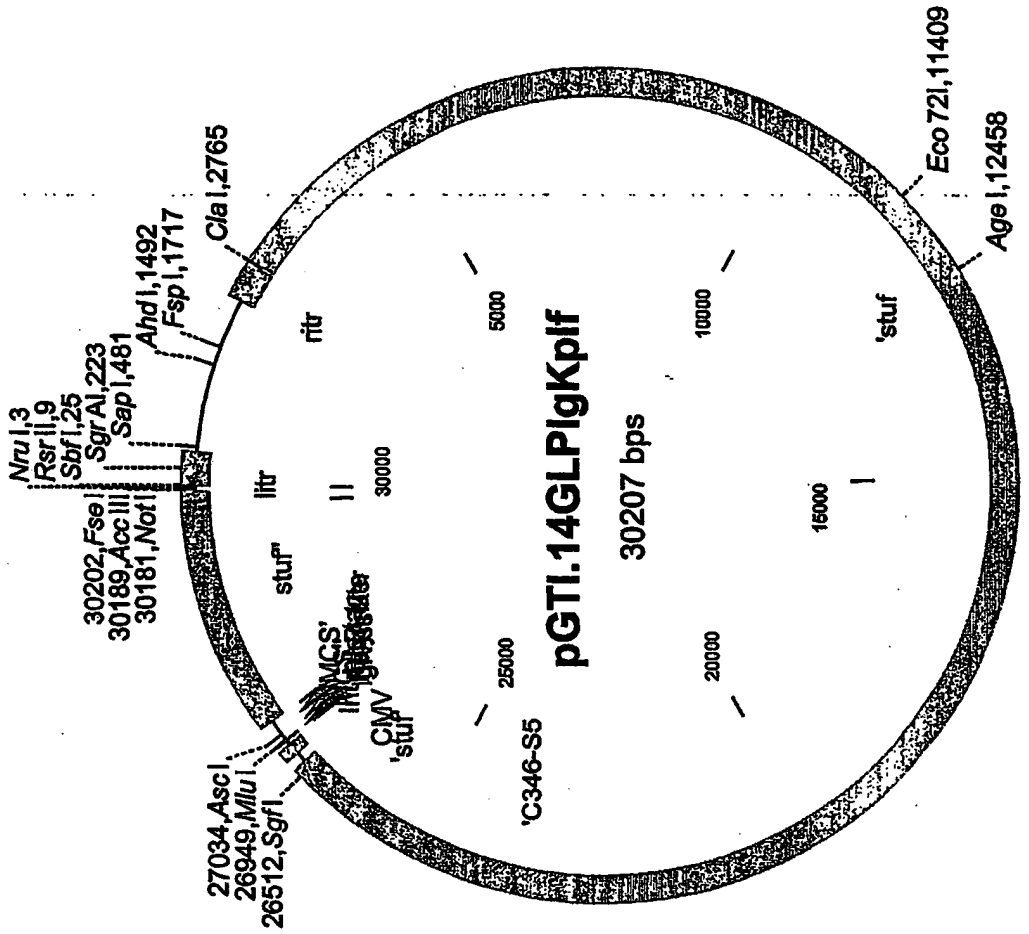

Figure 3E

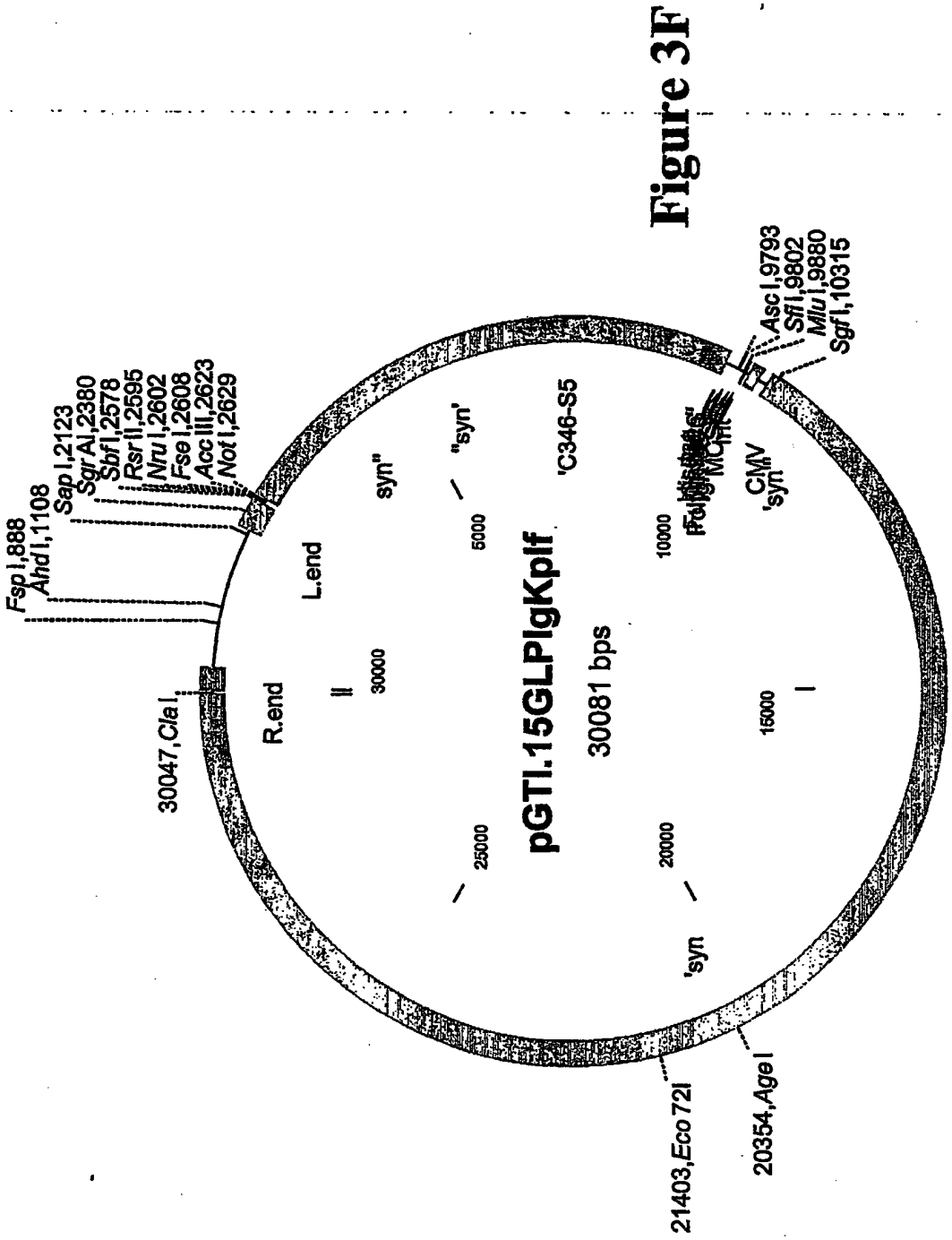

Figure 3F

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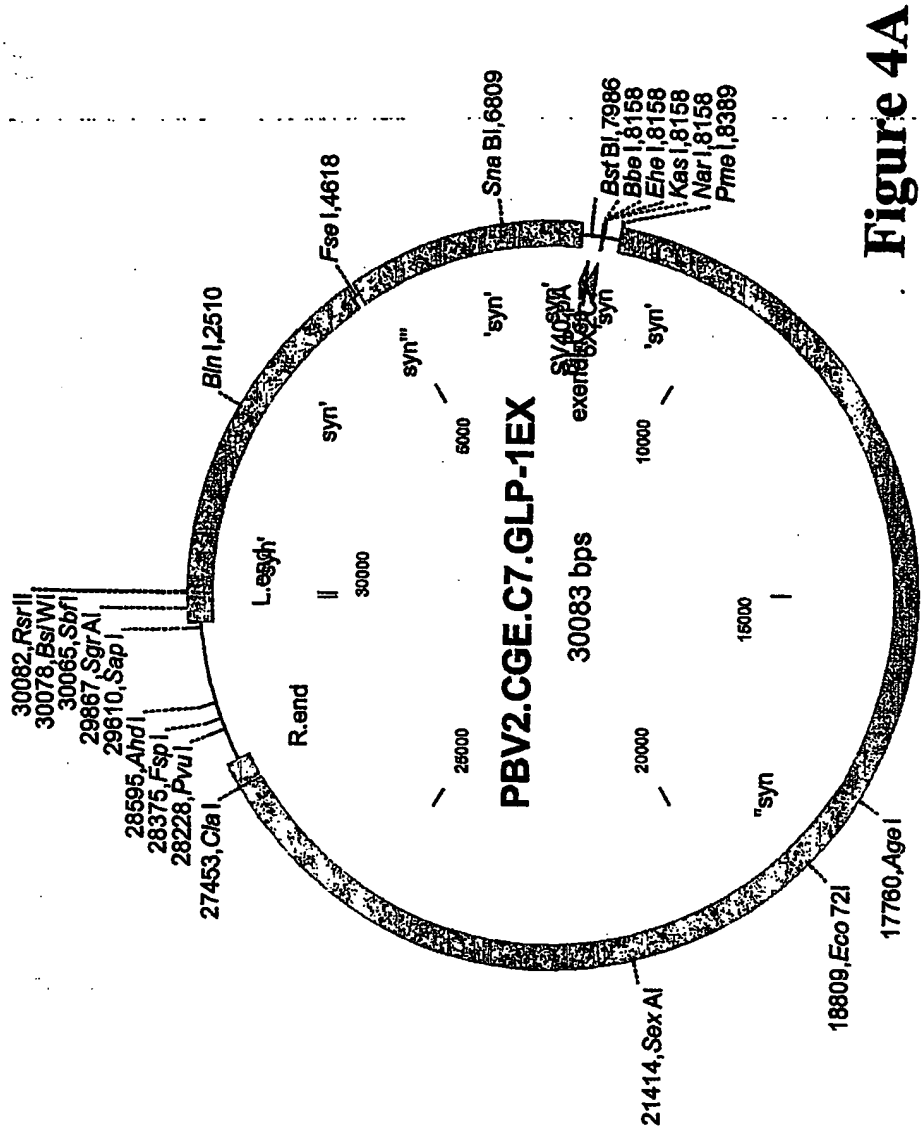

Figure 4A

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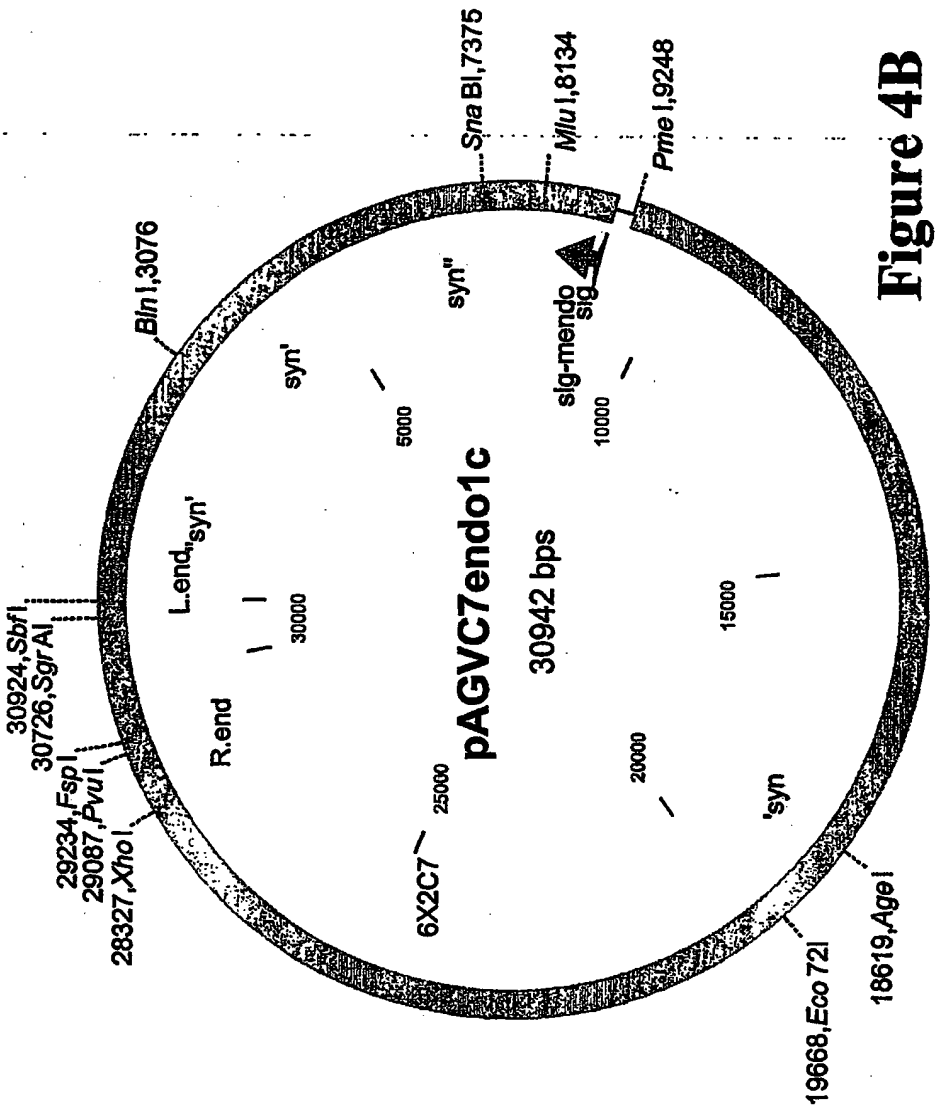

Figure 4B

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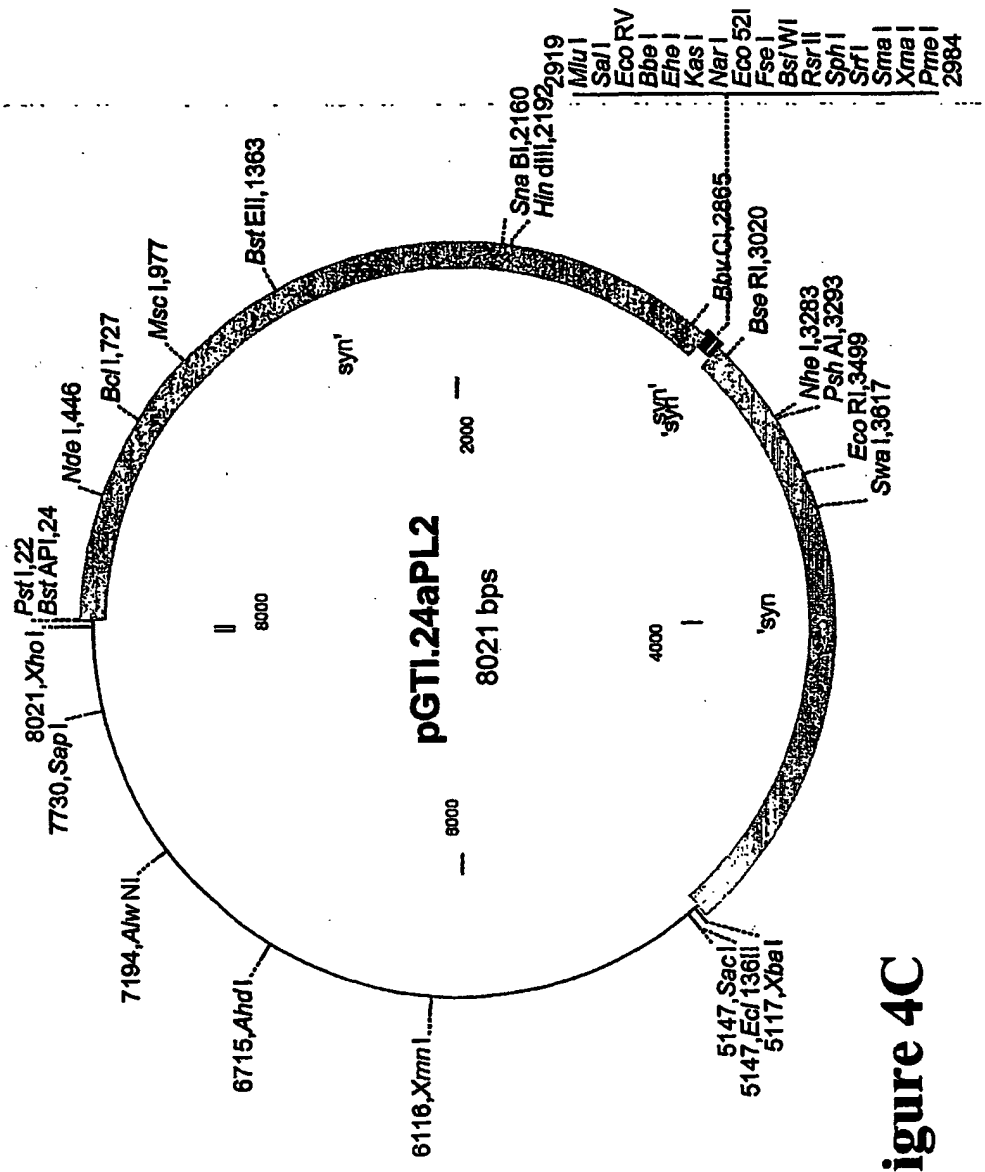

Figure 4C

Best Available Copy

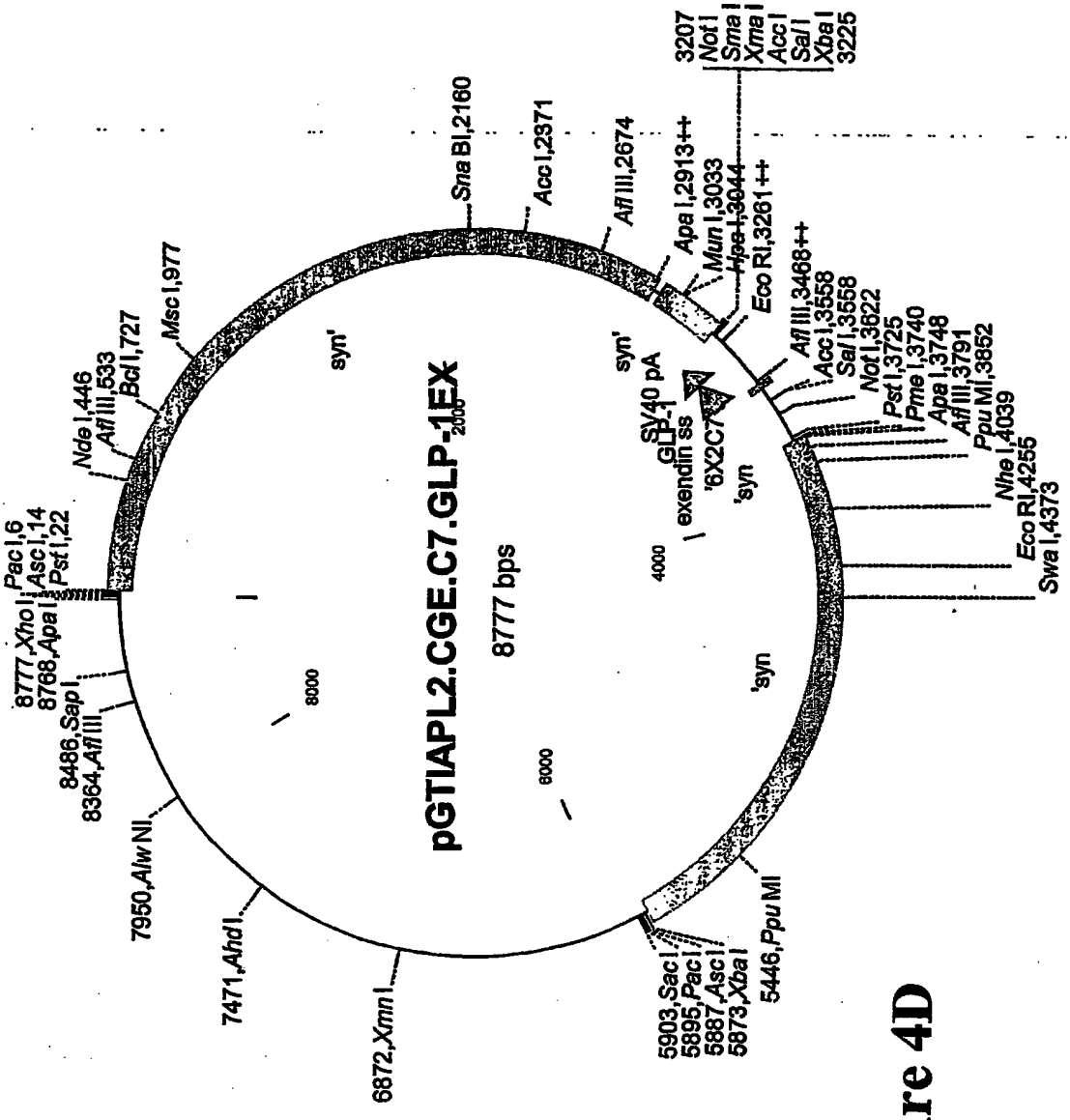

Figure 4D

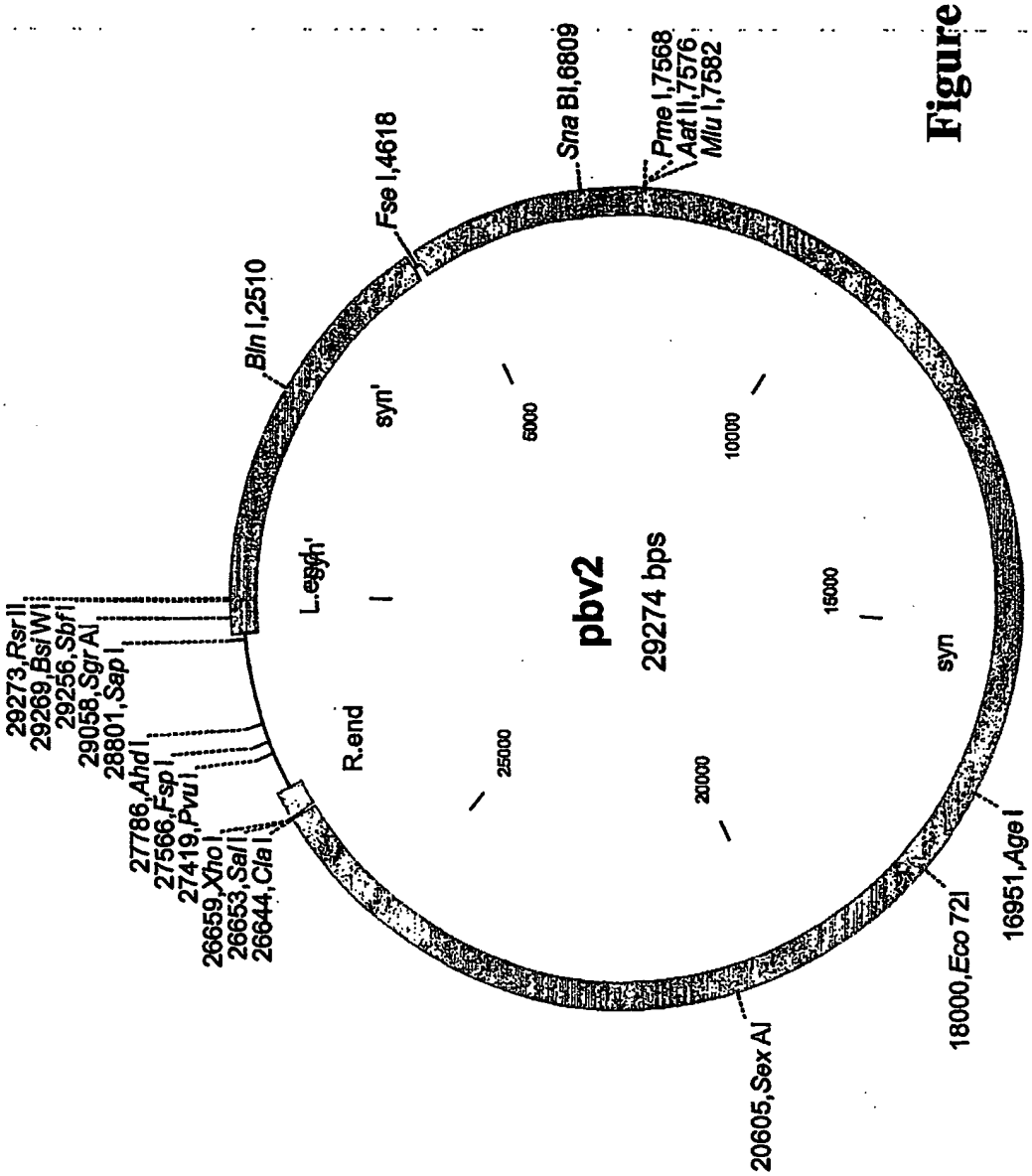

Figure 4E

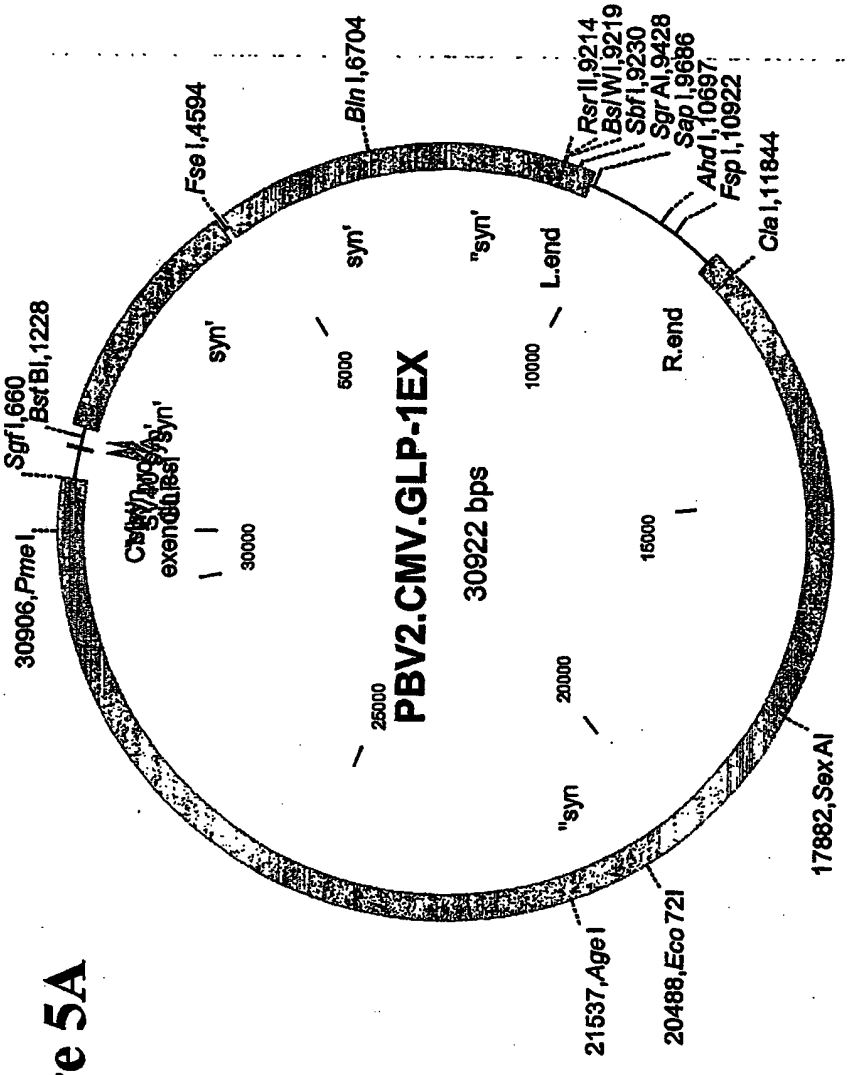

Figure 5A

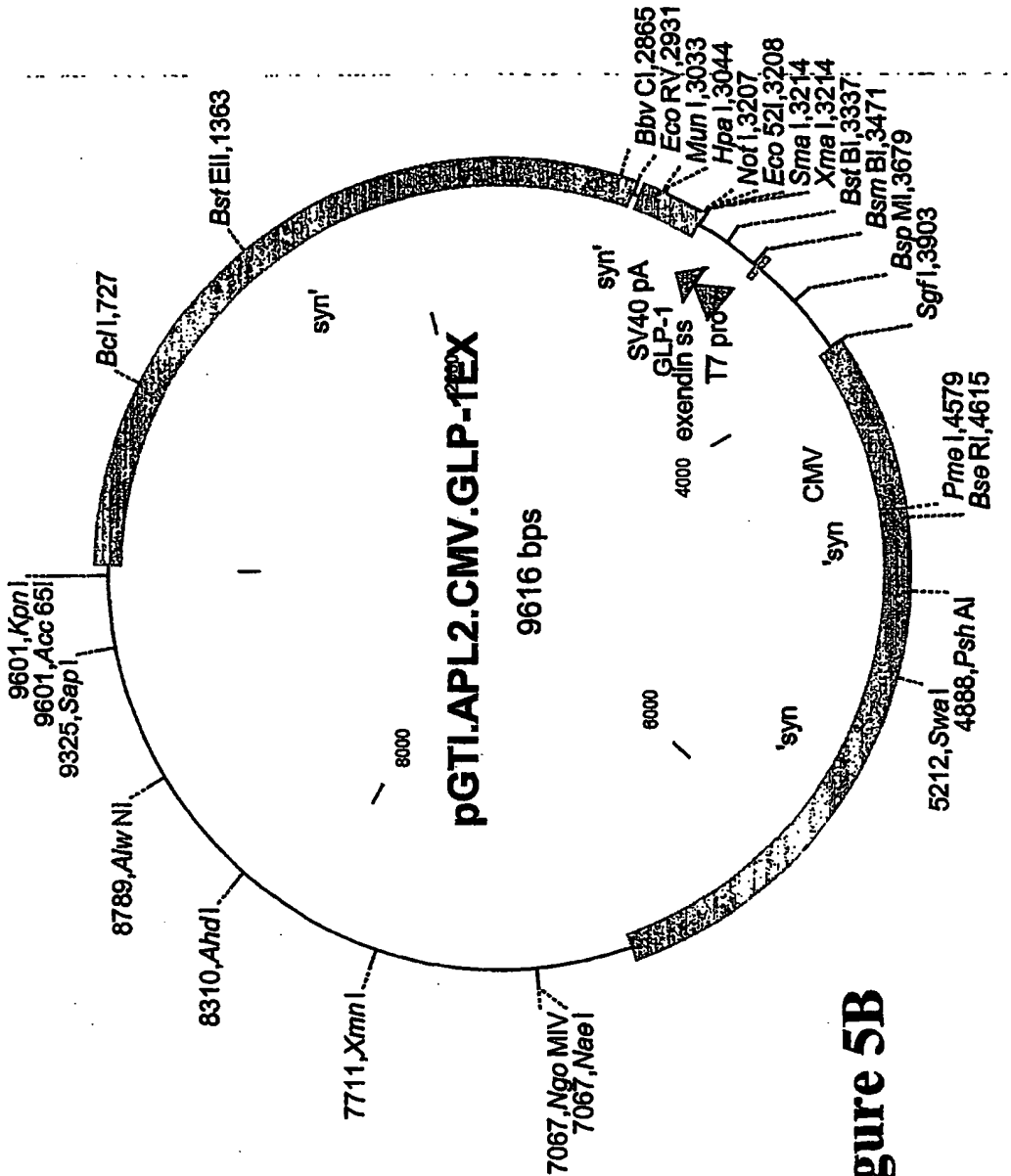

Figure 5B

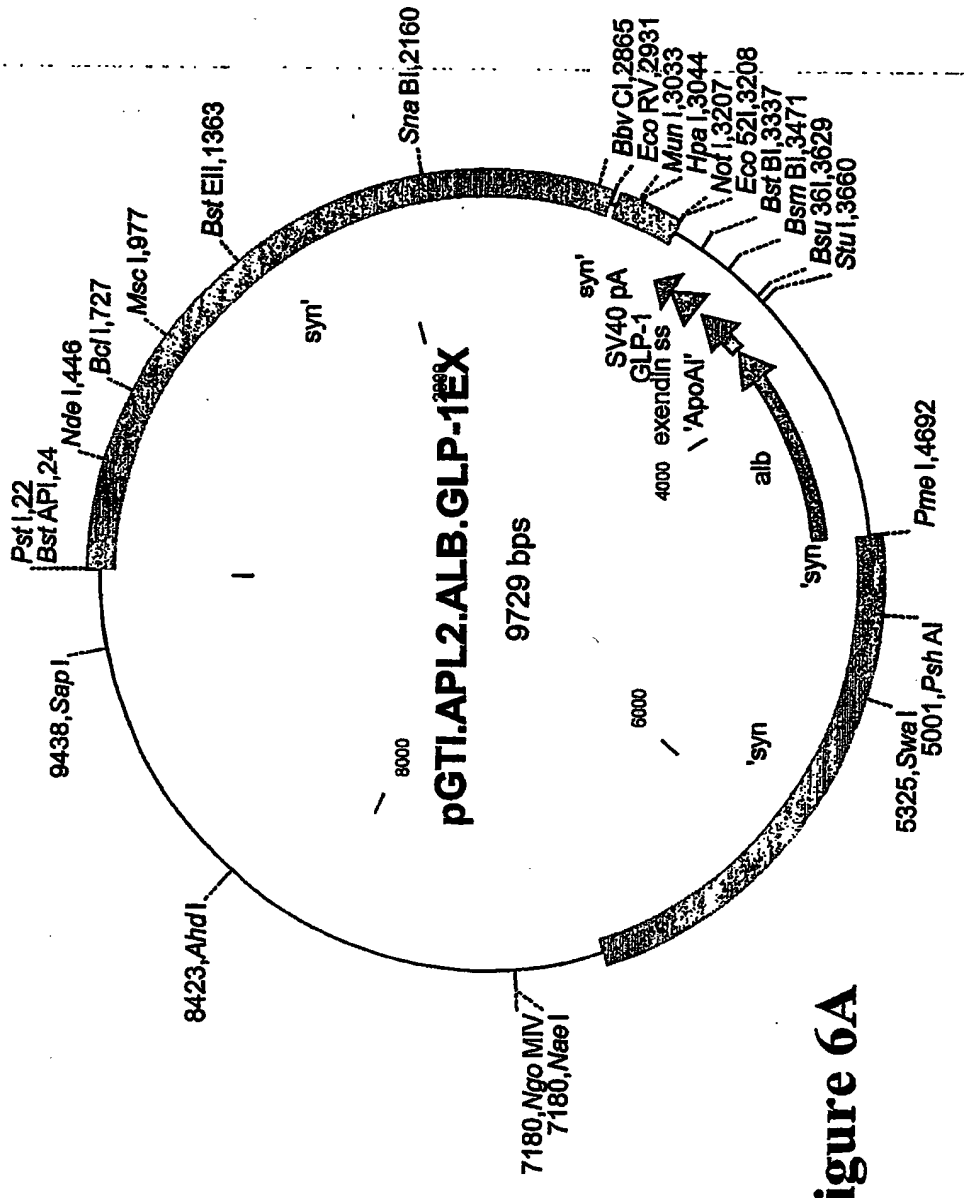

Figure 6A

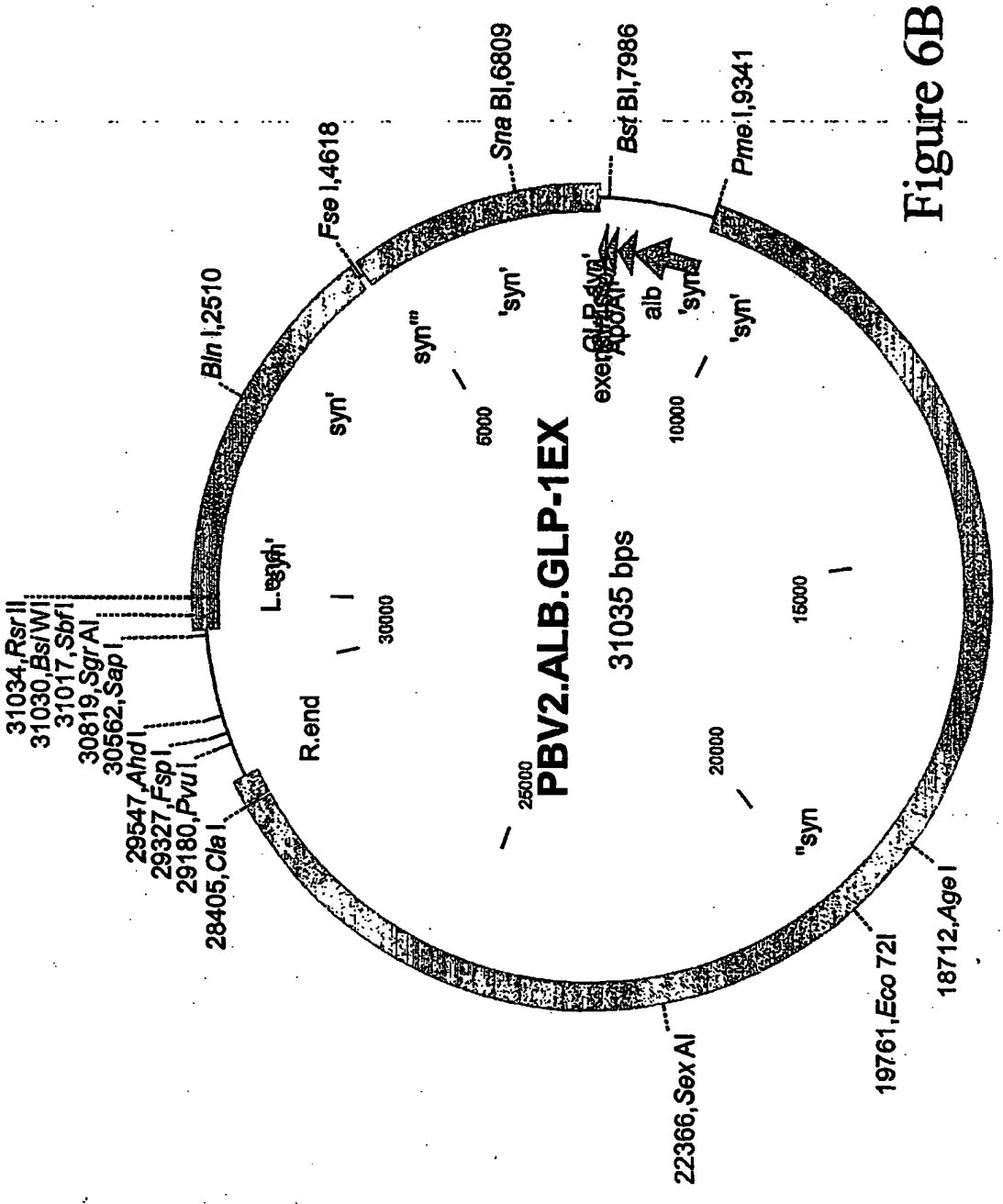

Figure 6B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/32051

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : A61K 48/00; C12P 21/00
US CL : 514/12, 44, 866; 435/69.1, 69.5, 252.3, 320.1, 325, 455
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/12, 44, 866; 435/69.1, 320.1, 325, 455

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2002/0065239 A1 (CAPLAN et al) 30 May 2001 (30.06.2001), [0013], [0082]-[0084], [0089], [0090].	1, 3, 4, 14, 15, 17
Y	US 6,110,707 (NEWGARD et al) 29 August 2000 (29.08.2000), column 4, line 13-18, column 6, line 23-54), column 44, line 14-column 47, line 31, column 50, line 5 to column 53, line 50,	1, 11, 13, 14, 15, 17,
Y, P	US 6,444,788 B1 (Staby et al) 03 September 2002 (03.09.2002), column 1, line 49-57, column 20, line 18-49.	1, 3, 4.

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"B" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 December 2002 (23.12.2002)	15 JAN 2003
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Maria Marvich, PhD Telephone No. (703) 605-1207

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

PCT/US02/32051

Continuation of B. FIELDS SEARCHED Item 3:
EAST databases USPAT, PGPUB, EPO, JPO, Derwent, IBM-IDN
STN databases Medline, CAPLUS

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/32051

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claim Nos.: 21-27 and 29-48
because they relate to subject matter not required to be searched by this Authority, namely:
Improper Multiple Dependent

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
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(10) International Publication Number
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(51) International Patent Classification[7]: C07K 5/06, 5/08, A61K 38/05, 38/06, A61P 25/00, 3/10

(21) International Application Number: PCT/EP02/08929

(22) International Filing Date: 9 August 2002 (09.08.2002)

(25) Filing Language: English

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(30) Priority Data:
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(71) Applicant *(for all designated States except US)*: **PROBIODRUG AG** [DE/DE]; Weinbergweg 22, 06120 Halle/Saale (DE).

(72) Inventors; and
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(74) Agents: **FORSTMEYER, Dietmar** et al.; Boeters & Bauer, Bereiteranger 15, 81541 München (DE).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

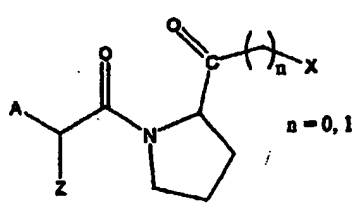
(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/033524 A2

(54) Title: PEPTIDYL KETONES AS INHIBITORS OF DPIV



(1)

n = 0, 1

(57) Abstract: The present invention relates to compounds of the general formula (I), and pharmaceutically acceptable salts thereof, to the use of the compounds for the treatment of impaired glucose tolerance, glucosuria, hyperlipidaemia, metabolic acidosis, diabetes mellitus, diabetic neuropathy and nephropathy and of sequelae caused by diabetes mellitus in mammals.

Peptidyl ketones as inhibitors of DPIV

The present invention relates to peptidyl ketones and salts thereof, hereinafter referred to as peptidyl ketones, and to the use of the compounds for the preparation of a medicament for the *in vivo* inhibition of DPIV or/and DPIV-like enzymes.

The invention relates especially to the use of the compounds for the preparation of a medicament for the treatment of impaired glucose tolerance, glucosuria, hyperlipidaemia, metabolic acidosis, diabetes mellitus, diabetic neuropathy and nephropathy and of sequelae caused by diabetes mellitus in mammals, for the treatment of metabolism-related hypertension and of cardiovascular sequelae caused by hypertension in mammals, for the prophylaxis or treatment of skin diseases and diseases of the mucosae, autoimmune diseases and inflammatory conditions, and for the treatment of psychosomatic, neuropsychiatric and depressive illnesses, such as anxiety, depression, sleep disorders, chronic fatigue, schizophrenia, epilepsy, nutritional disorders, spasm and chronic pain.

Dipeptidyl peptidase IV (DPIV) is a post-proline (to a lesser extent post-alanine, post-serine or post-glycine) cleaving serine protease found in various tissues of the body including kidney, liver, and intestine, where it removes dipeptides from the N-terminus of biologically active peptides with a high specificity when proline or alanine form the residues that are adjacent to the N-terminal amino acid in their sequence.

The term DPIV-like enzymes relates to structurally and/or functionally DPIV/CD26-related enzyme proteins (Sedo & Malik, Dipeptidyl peptidase IV-like molecules: homologous proteins or homologous activities? Biochimica et Biophysica Acta 2001, 36506: 1-10). In essence, this small group of enzymes has evolved during evolution to release H-Xaa-Pro-Dipeptides and H-Xaa-Ala-Dipeptides from the N-terminus of oligo- or polypeptides. They show the common feature, that they accomodate in the Pro-position also Ala, Ser, Thr and other amino

acids with small hydrophobic side-chains as Gly or Val. The hydrolytic efficacy is ranked Pro>Ala» Ser, Thr » Gly, Val. Same proteins have been only available in such small quantities that only the post-Pro or post-Ala cleavage could be established. While the proteins: DPIV, DP II, FAPα (Seprase), DP 6, DP 8 and DP 9 are structurally related and show a high sequence homology, attractin is an extraordinary functional DPIV-like enzyme, characterized by a similar activity and inhibitory pattern.

Further DPIV-like enzymes are disclosed in WO 01/19866, WO 02/34900 and WO02/31134. WO 01/19866 discloses novel human dipeptidyl aminopeptidase 8 (DPP8) with structural und functional similarities to DPIV and fibroblast activation protein (FAP). WO 02/34900 discloses a novel dipeptidyl peptidase 9 (DPP9) with significant homology to the amino acid sequences of DPIV and DPP8. WO 02/31134 discloses three DPIV-like enzymes, DPRP1, DPRP2 and DPRP3. Sequence analysis revealed that DPRP1 is identical to DPP8 as disclosed in WO 01/19866, that DPRP2 is identical to DPP9 and that DPRP3 is identical to KIAA1492 as disclosed in WO 02/04610.

Likewise, it has been discovered that DPIV is responsible for inactivating glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide also known as gastric-inhibitory peptide (GIP). Since GLP-1 is a major stimulator of pancreatic insulin secretion and has direct beneficial effects on glucose disposal, in WO 97/40832 and US 6,303,661 inhibition of DPIV and DPIV-like enzyme activity was shown to represent an attractive approach for treating non-insulin-dependent diabetes mellitus (NIDDM).

The reduction of such DP IV and DPIV-like enzyme activity for cleaving such substrates *in vivo* can serve to suppress undesirable enzyme activity effectively both under laboratory conditions and in pathological conditions of mammals. For example, *Diabetes mellitus* type II (also diabetes of old age) is based upon reduced insulin secretion or disturbances in receptor function which are founded *inter alia*

upon proteolytically determined abnormalities in the concentration of the incretins.

Hyperglycaemia and its associated causes and sequelae (also *Diabetes mellitus*) are treated according to the current state of the art by administering insulin (for example material isolated from bovine pancreas or also material obtained by genetic engineering) to those affected, in various forms of administration. All of the previously known methods and also more modern methods are characterised by high expenditure on materials, high costs and often by crucial impairment of the patient's life quality. The classical method (daily *i.v.* insulin injection, customary since the thirties) treats the acute symptoms of the disease but leads, after prolonged use, to *inter alia* severe vascular changes (arteriosclerosis) and nerve damage.

It is known that DPIV-Inhibitors may be useful for the treatment of impaired glucose tolerance and diabetes mellitus (International Patent Application, Publication Number WO 99/61431, Pederson RA et al, Diabetes. 1998 Aug; 47(8):1253-8 and Pauly RP et al, Metabolism 1999 Mar; 48(3):385-9). In particular WO 99/61431 discloses DPIV-Inhibitors comprising an amino acid residue and a thiazolidine or pyrrolidine group, and salts thereof, especially L-*threo*-isoleucyl thiazolidine, L-*allo*-isoleucyl thiazolidine, L-*threo*-isoleucyl pyrrolidine, L-*allo*-isoleucyl thiazolidine, L-*allo*-isoleucyl pyrrolidine, and salts thereof.

Further examples of low molecular weight dipeptidyl peptidase IV inhibitors are agents such as tetrahydroisoquinolin-3-carboxamide derivatives, N-substituted 2-cyanopyrroles and –pyrrolidines, N-(N'-substituted glycyl)–2-cyanopyrrolidines, N-(substituted glycyl)-thiazolidines, N-(substituted glycyl)-4-cyanothiazolidines, amino-acyl-borono-prolyl-inhibitors and cyclopropyl-fused pyrrolidines. Inhibitors of dipeptidyl peptidase IV are described in US 6,011,155; US 6,107,317; US 6,110,949; US 6,124,305; US 6,172,081; WO 99/61431, WO 99/67278, WO 99/67279, DE 198 34 591, WO 97/40832, DE 196 16 486 C 2, WO 98/19998, WO 00/07617, WO 99/38501, WO 99/46272, WO 99/38501, WO 01/68603, WO 01/40180, WO 01/81337, WO 01/81304, WO 01/55105, WO 02/02560 and WO

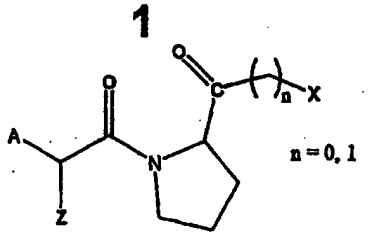
02/14271, the teachings of which are herein incorporated by reference in their entirety concerning these inhibitors, their uses, definition and their production.

More recently, the installation of subcutaneous depot implants (the insulin is released in metered amounts, and daily injections are unnecessary) and the implantation (transplantation) of intact Langerhans cells into the dysfunctional pancreas gland or other organs and tissues have been proposed. Such transplantation is complicated from a technical point of view. It furthermore represents risky surgical intervention in the recipient and, in the case of cell transplantation, also requires methods of suppressing or by-passing the immune system.

The problem of the invention is therefore to provide new compounds for the treatment of, for example, impaired glucose tolerance, glucosuria, hyperlipidaemia, metabolic acidosis, diabetes mellitus, diabetic neuropathy and nephropathy and of sequelae caused by diabetes mellitus in mammals, metabolism-related hypertension and cardiovascular sequelae caused by hypertension in mammals, for the prophylaxis or treatment of skin diseases and diseases of the mucosae, autoimmune diseases and inflammatory conditions, and for the treatment of psycho-somatic, neuropsychiatric and depressive illnesses, such as anxiety, depression, sleep disorders, chronic fatigue, schizophrenia, epilepsy, nutritional disorders, spasm and chronic pain, and a simple method for the treatment of those diseases.

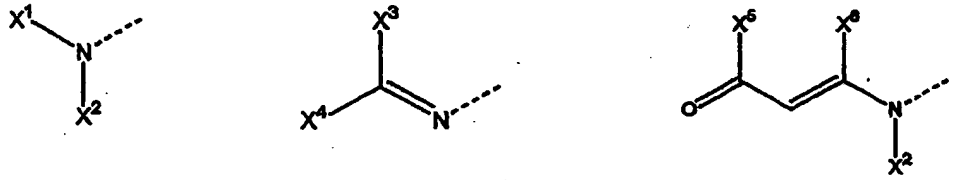
That problem is solved according to the invention by providing compounds of the general formula 1 and pharmaceutically acceptable salts thereof, including all stereoisomers:

1



and pharmaceutically acceptable salts thereof, wherein:

A is selected from the following structures:



wherein

X^1 is H or an acyl or oxycarbonyl group including an amino acid residue, a N-protected amino acid residue, a peptide residue or a N-protected peptide residue,

X^2 is H, $-(CH)_m-NH-C_5H_3N-Y$ with m = 2-4 or $-C_5H_3N-Y$ (a divalent pyridyl residue) and Y is selected from H, Br, Cl, I, NO_2 or CN,

X^3 is H or selected from an alkyl-, alkoxy-, halogen-, nitro-, cyano- or carboxy- substituted phenyl or from an alkyl-, alkoxy-, halogen-, nitro-, cyano- or carboxy- substituted pyridyl residue,

X^4 is H or selected from an alkyl-, alkoxy-, halogen-, nitro-, cyano- or carboxy- substituted phenyl or from an alkyl-, alkoxy-, halogen-, nitro-, cyano- or carboxy- substituted pyridyl residue,

X^5 is H or an alkyl, alkoxy or phenyl residue,

X^6 is H or an alkyl residue,

for n = 1

X is selected from: H, OR^2, SR^2, NR^2R^3, $N^+R^2R^3R^4$, wherein:

R^2 stands for acyl residues, which are optionally substituted with alkyl, cycloalkyl, aryl or heteroaryl residues, or for amino acid residues or peptidic residues, or alkyl residues, which are optionally substituted with alkyl,

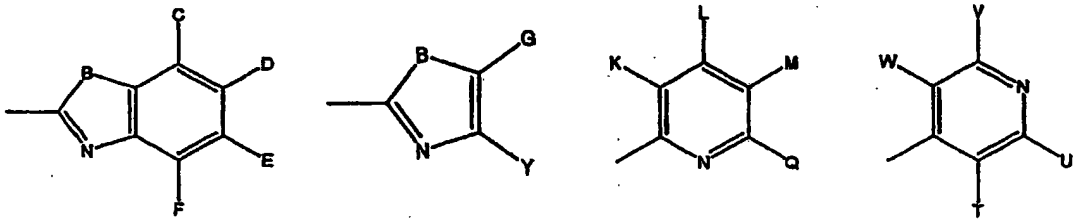
cycloalkyl, aryl or heteroaryl residues,

R^3 stands for alkyl or acyl residues, wherein R^2 and R^3 may be part of a saturated or unsaturated carbocyclic or heterocyclic ring,

R^4 stands for alkyl residues, wherein R^2 and R^4 or R^3 and R^4 may be part of a saturated or unsaturated carbocyclic or heterocyclic ring,

for n = 0

X is selected from:



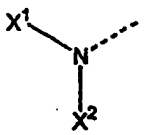
wherein

B stands for: O, S or NR^5, wherein R^5 is H, alkyl or acyl,

C, D, E, F, G, Y, K, L, M, Q, T, U, V and W are independently selected from alkyl and substituted alkyl residues, oxyalkyl, thioalkyl, aminoalkyl, carbonylalkyl, acyl, carbamoyl, aryl and heteroaryl residues, and

Z is selected from H, or a branched or straight chain alkyl residue from C_1-C_8, a branched or straight chain alkenyl residue from C_2-C_8, a cycloalkyl residue from C_3-C_8, a cycloalkenyl residue from C_5-C_7, an aryl or heteroaryl residue, or a side chain selected from all side chains of all natural amino acids or derivatives thereof.

In preferred compounds of formula 1, A is



wherein

X^1 is H or an acyl or oxycarbonyl group including an amino acid residue, N-acylated amino acid residue, a peptide residue from di- to pentapeptides, preferably a dipeptide residue, or a N-protected peptide residue from di- to pentapeptides, preferably a N-protected dipeptide residue,

X^2 is H, $-(CH)_m-NH-C_5H_3N-Y$ with m = 2-4 or $-C_5H_3N-Y$ (a divalent pyridyl residue) and Y is selected from H, Br, Cl, I, NO_2 or CN,

for n = 1

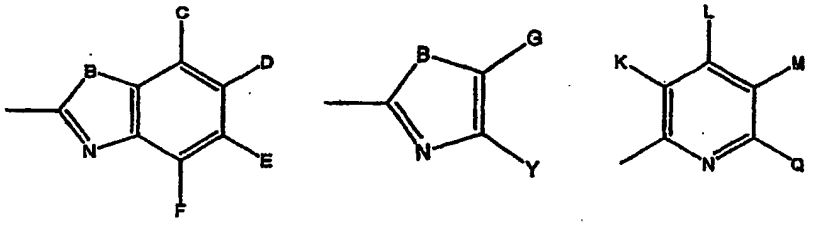
X is preferably selected from: H, OR^2, SR^2, NR^2R^3, wherein:

R^2 stands for acyl residues, which are optionally substituted with alkyl, cycloalkyl, aryl or heteroaryl residues, or for amino acid residues or peptidic residues, or alkyl residues, which are optionally substituted with alkyl, cycloalkyl, aryl or heteroaryl residues,

R^3 stands for alkyl or acyl residues, wherein R^2 and R^3 may be part of a saturated or unsaturated carbocyclic or heterocyclic ring,

for n = 0

X is preferably selected from:



wherein

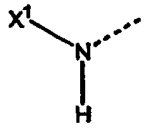
B stands for: O, S or NR^5, wherein R^5 is H, alkyl or acyl,

C, D, E, F, G, Y, K, L, M and Q are independently selected from alkyl and substituted alkyl residues, oxyalkyl, thioalkyl, aminoalkyl, carbonylalkyl, acyl,

carbamoyl, aryl and heteroaryl residues, and

Z is selected from H, or a branched or straight chain alkyl residue from C_1-C_9, preferably C_2-C_6, a branched or straight chain alkenyl residue from C_2-C_9, a cycloalkyl residue from C_3-C_8, a cycloalkenyl residue from C_5-C_7, an aryl or heteroaryl residue, or a side chain selected from all side chains of all natural amino acids or derivatives thereof.

In more preferred compounds of formula 1, A is



wherein

X^1 is H or an acyl or oxycarbonyl group including an amino acid residue, N-acylated amino acid residue or a peptide residue from di- to pentapeptides, preferably a dipeptide residue, or a N-protected peptide residue from di- to pentapeptides, preferably a N-protected dipeptide residue

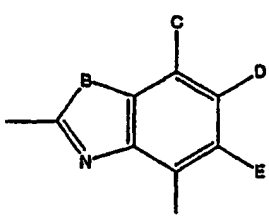
for n = 1,

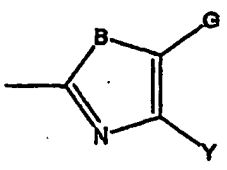
X is preferably selected from: H, OR^2, SR^2, wherein:

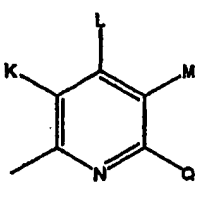
R^2 stands for acyl residues, which are optionally substituted with alkyl or aryl residues,

for n = 0

X is preferably selected from:







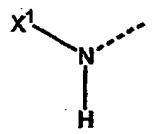
wherein

B stands for: O, S or NR^5, wherein R^5 is H, alkyl or acyl,

C, D, E, F, G, Y, K, L, M and Q, are independently selected from alkyl and substituted alkyl residues, oxyalkyl, thioalkyl, aminoalkyl, carbonylalkyl, acyl, carbamoyl, aryl and heteroaryl residues, and

Z is selected from H, or a branched or straight chain alkyl residue from C_1-C_9, preferably C_2- C_8, a branched or straight chain alkenyl residue from C_2-C_9, a cycloalkyl residue from C_3-C_8, a cycloalkenyl residue from C_5-C_7, an aryl or heteroaryl residue, or a side chain selected from all side chains of all natural amino acids or derivatives thereof.

In most preferred compounds of formula 1, A is



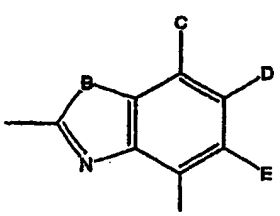
wherein

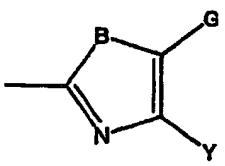
X^1 is H or an acyl or oxycarbonyl group including an amino acid residue, N-acylated amino acid residue or a dipeptide residue, containing a Pro or Ala in the penultimate position, or a N-protected dipeptide residue containing a Pro or Ala in the penultimate position,for n = 1,

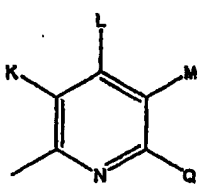
X is H,

for n = 0

X is preferably selected from:







wherein

B stands for: O or S, most preferably for S

C, D, E, F, G, Y, K, L, M, Q, are H and

Z is selected from H, or a branched or straight chain alkyl residue from C_3-C_5, a branched or straight chain alkenyl residue from C_2-C_9, a cycloalkyl residue from C_5-C_7, a cycloalkenyl residue from C_5-C_7, an aryl or heteroaryl residue, or a side chain selected from all side chains of all natural amino acids or derivatives thereof.
Most preferred for Z is H.

According to a preferred embodiment the acyl groups are C1-C6-acyl groups.
According to a further preferred embodiment the alk(yl) groups are C1-C6-alk(yl) groups, which may be branched or unbranched.
According to a further preferred embodiment the alkoxy groups are C1-C6-alkoxy groups.
According to a further preferred embodiment the aryl residues are C5-C12 aryl residues that have optionally fused rings.
According to a further preferred embodiment the cycloalkyl residues (carbocycles) are C3-C8-cycloalkyl residues.
According to a further preferred embodiment the heteroaryl residues are C4-C11 aryl residues that have optionally fused rings and, in at least one ring, additionally from 1 to 4 preferably 1 or 2 hetero atoms, such as O, N and/or S.
According to a further preferred embodiment peptide residues are corresponding residues containing from 2 to 50 amino acids.
According to a further preferred embodiment the heterocyclic residues are C2-C7-cycloalkyl radicals that additionally have from 1 to 4, preferably 1 or 2 hetero atoms, such as O, N and/or S.
According to a further preferred embodiment the carboxy groups are C1 – C6 carboxy groups, which may be branched or unbranched.
According to a further preferred embodiment the oxycarbonyl groups are groups of

the formula –O-$(CH_2)_{1-6}$COOH.

The amino acids can be any natural or synthetic amino acid, preferably natural alpha amino acids.

Examples of amino acids which can be used in the present invention are L and D-amino acids, N-methyl-amino-acids; *allo*- and *threo*-forms of Ile and Thr, which can, e.g. be α-, β- or ω-amino acids, whereof α-amino acids are preferred.

Examples of amino acids are:
aspartic acid (Asp), glutamic acid (Glu), arginine (Arg), lysine (Lys), histidine (His), glycine (Gly), serine (Ser) and cysteine (Cys), threonine (Thr), asparagine (Asn), glutamine (Gln), tyrosine (Tyr), alanine (Ala), proline (Pro), valine (Val), isoleucine (Ile), leucine (Leu), methionine (Met), phenylalanine (Phe), tryptophan (Trp), hydroxyproline (Hyp), beta-alanine (beta-Ala), 2-amino octanoic acid (Aoa), azetidine-(2)-carboxylic acid (Ace), pipecolic acid (Pip), 3-amino propionic, 4-amino butyric and so forth, alpha-aminoisobutyric acid (Aib), sarcosine (Sar), ornithine (Orn), citrulline (Cit), homoarginine (Har), t-butylalanine (*t*-butyl-Ala), t-butylglycine (*t*-butyl-Gly), N-methylisoleucine (N-MeIle), phenylglycine (Phg), cyclohexylalanine (Cha), norleucine (Nle), cysteic acid (Cya) and methionine sulfoxide (MSO), Acetyl-Lys, modified amino acids such as phosphoryl-serine (Ser(P)), benzyl-serine (Ser(Bzl)) and phosphoryl-tyrosine (Tyr(P)), 2-aminobutyric acid (Abu), aminoethylcysteine (AECys), carboxymethylcysteine (Cmc), dehydroalanine (Dha), dehydroamino-2-butyric acid (Dhb), carboxyglutaminic acid (Gla), homoserine (Hse), hydroxylysine (Hyl), *cis*-hydroxyproline (*cis*Hyp), *trans*-hydroxyproline (*trans*Hyp), isovaline (Iva), pyroglutamic acid (Pyr), norvaline (Nva), 2-aminobenzoic acid (2-Abz), 3- aminobenzoic acid (3-Abz), 4- aminobenzoic acid (4-Abz), 4-(aminomethyl)benzoic acid (Amb), 4-(aminomethyl)cyclohexanecarboxylic acid (4-Amc), Penicillamine (Pen), 2-Amino-4-cyanobutyric acid (Cba), cycloalkane-carboxylic aicds.

Examples of ω-amino acids are e.g.: 5-Ara (aminoraleric acid), 6-Ahx (aminohexanoic acid), 8-Aoc (aminooctanoic aicd), 9-Anc (aminovanoic aicd), 10-Adc (aminodecanoic acid), 11-Aun (aminoundecanoic acid), 12-Ado (aminododecanoic acid).

Further amino acids are: indanylglycine (Igl), indoline-2-carboxylic acid (Idc), octahydroindole-2-carboxylic acid (Oic), diaminopropionic acid (Dpr), diaminobutyric acid (Dbu), naphtylalanine (1-Nal), (2-Nal), 4-aminophenylalanin (Phe(4-NH_2)), 4-benzoylphenylalanine (Bpa), diphenylalanine (Dip), 4-bromophenylalanine (Phe(4-Br)), 2-chlorophenylalanine (Phe(2-Cl)), 3-chlorophenylalanine (Phe(3-Cl)), 4-chlorophenylalanine (Phe(4-Cl)), 3,4-chlorophenylalanine (Phe (3,4-Cl_2)), 3- fluorophenylalanine (Phe(3-F)), 4-fluorophenylalanine (Phe(4-F)), 3,4- fluorophenylalanine (Phe(3,4-F_2)), pentafluorophenylalanine (Phe(F_5)), 4-guanidinophenylalanine (Phe(4-guanidino)), homophenylalanine (hPhe), 3-jodophenylalanine (Phe(3-J)), 4 jodophenylalanine (Phe(4-J)), 4-methylphenylalanine (Phe(4-Me)), 4-nitrophenylalanine (Phe-4-NO_2)), biphenylalanine (Bip), 4-phosphonomehtylphenylalanine (Pmp), cyclohexyglycine (Ghg), 3-pyridinylalanine (3-Pal), 4-pyridinylalanine (4-Pal), 3,4-dehydroproline (A-Pro), 4-ketoproline (Pro(4-keto)), thioproline (Thz), isonipecotic acid (Inp), 1,2,3,4,-tetrahydroisoquinolin-3-carboxylic acid (Tic), propargylglycine (Pra), 6-hydroxynorleucine (NU(6-OH)), homotyrosine (hTyr), 3-jodotyrosine (Tyr(3-J)), 3,5-dijodotyrosine (Tyr(3,5-J_2)), d-methyl-tyrosine (Tyr(Me)), 3-NO_2-tyrosine (Tyr(3-NO_2)), phosphotyrosine (Tyr(PO_3H_2)), alkylglycine, 1-aminoindane-1-carboxy acid, 2-aminoindane-2-carboxy acid (Aic), 4-amino-methylpyrrol-2-carboxylic acid (Py), 4-amino-pyrrolidine–2-carboxylic acid (Abpc), 2-aminotetraline-2-carboxylic acid (Atc), diaminoacetic acid (Gly(NH_2)), diaminobutyric acid (Dab), 1,3-dihydro-2H-isoinole-carboxylic acid (Disc), homocylcohexylalanin (hCha), homophenylalanin (hPhe oder Hof), *trans*-3-phenyl-azetidine-2-carboxylic acid, 4-phenyl-pyrroljdine-2-carboxylic acid, 5-phenyl-pyrrolidine-2-carboxylic acid, 3-pyridylalanine (3-Pya), 4-pyridylalanine (4-Pya), styrylalanine, tetrahydroisoquinoline-1-carboxylic acid (Tiq), 1,2,3,4-tetrahydronorharmane-3-carboxylic acid (Tpi), ß-(2-thienryl)-alanine (Tha)

Upon - preferably oral - administration of those compounds to a mammal, the endogenous (or additionally exogenously administered) insulinotropic peptides GIP_{1-42} and GLP-1_{7-36} (or GLP-1_{7-37} or analogues thereof), for example, are broken down to a lesser degree by DPIV or DPIV-like enzymes and hence the reduction in the concentration of those peptide hormones and their analogues is reduced or delayed. The invention is based, therefore, on the finding that a reduction of the DPIV or DPIV-like enzyme activity in the bloodstream results in influencing of the blood sugar level.

The oral administration of the high-affinity, low-molecular-weight enzyme inhibitors of the invention is a more cost-effective alternative, for example, to invasive surgical techniques in the treatment of pathological symptoms. By chemical design of stability, transport and clearance properties their mode of action can be modified and matched to individual characteristics.

The salts of the compounds of the invention may, assuming that they have basic properties, be in the form of inorganic or organic salts.

The compounds of the present invention can be converted into and used as acid addition salts, especially pharmaceutically acceptable acid addition salts. The pharmaceutically acceptable salt generally takes a form in which a basic side chain is protonated with an inorganic or organic acid. Representative organic or inorganic acids include hydrochloric, hydrobromic, perchloric, sulfuric, nitric, phosphoric, acetic, propionic, glycolic, lactic, succinic, maleic, fumaric, malic, tartaric, citric, benzoic, mandelic, methanesulfonic, hydroxyethanesulfonic, benzenesulfonic, oxalic, pamoic, 2-naphthalenesulfonic, p-toulenesulfonic, cyclohexanesulfamic, salicylic, saccharinic or trifluoroacetic acid. All pharmaceutically acceptable acid addition salt forms of the compounds of the present invention are intended to be embraced by the scope of this invention.

In view of the close relationship between the free compounds and the compounds in the form of their salts, whenever a compound is referred to in this context, a corresponding salt is also intended, provided such is possible or appropriate under the circumstances.

Where the compounds according to this invention have at least one chiral center, they may accordingly exist as enantiomers. Where the compounds possess two or more chiral centers, they may additionally exist as diastereomers. It is to be understood that all such isomers and mixtures thereof are encompassed within the scope of the present invention. Furthermore, some of the crystalline forms of the compounds may exist as polymorphs and as such are intended to be included in the present invention. In addition, some of the compounds may form solvates with water (i.e. hydrates) or common organic solvents, and such solvates are also intended to be encompassed within the scope of this invention.

The compounds, including their salts, can also be obtained in the form of their hydrates, or include other solvents used for their crystallization.

The invention accordingly relates to effectors, especially inhibitors of dipeptidyl peptidase IV (DPIV) and DPIV-like enzyme activity and to their use for lowering the blood sugar level below the glucose concentration characteristic of hyperglycaemia in the serum of a mammal. The invention relates especially to the use of the compounds of the invention for modulating DPIV and DPIV-like enzyme activity in order to prevent or alleviate pathological metabolic abnormalities of mammals, such as, for example, impaired glucose tolerance, glucosuria, hyperlipidaemia, metabolic acidosis, diabetes mellitus, diabetic neuropathy and nephropathy, and sequelae caused by diabetes mellitus in mammals. The invention further relates to the use of the compounds of the invention for modulating DPIV and DPIV-like enzyme activity in order to prevent or alleviate neurodegenerative diseases and high blood pressure. In the case of chronic administration of the compounds of the invention, the invention relates to the improvement of signal action at the cells of the islets of

Langerhans and of insulin sensitivity in the peripheral tissue in the postprandial phase.

The invention further relates to the use of the compounds of the invention for the chronic treatment of chronic metabolic diseases in humans; for the chronic treatment of chronically impaired glucose tolerance, chronic glucosuria, chronic hyperlipidaemia, chronic metabolic acidosis, chronic diabetes mellitus, chronic diabetic neuropathy and nephropathy and of chronic sequelae caused by diabetes mellitus, chronic neurodegenerative diseases and chronic disturbance of signal action at the cells of the islets of Langerhans and chronic insulin sensitivity in the peripheral tissue in the postprandial phase of mammals; for the chronic treatment of chronic metabolism-related hypertension and of chronic cardiovascular sequelae caused by hypertension in mammals; for the chronic treatment of chronic psychosomatic, chronic neuropsychiatric and depressive illnesses, such as chronic anxiety, chronic depression, chronic sleep disorders, chronic fatigue, chronic schizophrenia, chronic epilepsy, chronic nutritional disorders, spasm and chronic pain.

The compounds of the present invention act as prodrugs of DPIV-inhibitors. According to the invention, the compounds can be used as effectors, especially as inhibitors of DPIV and DPIV-like enzymes and it is possible to define the site of their action, the time of onset of their action and the duration of action precisely.

Upon administration, the compounds of the invention are cleaved, for example by suitable enzymes, and the active inhibitors are released. The active inhibitors can be released both by chemical and enzymatic mechanisms. For example, esterases, proteases and peptidases serve to release the active inhibitors from the compounds according to the invention. Such esterases, proteases, etc. are disclosed, for example, in WO 97/45117, US 5433955, US 5614379 and US 5624894. Preferred proteases are aminopeptidases, dipeptidyl aminopeptidases, endoproteases, and endopeptidases. Especially preferred proteases for the release of the active

inhibitors from the compounds of the present invention are aminopeptidase N, aminopeptidase P, pyroglutaminyl aminopeptidase,dipeptidyl pepitdase IV and dipeptidyl pepitdase IV-like enzymes.

The released active inhibitors can interact with the DPIV and DPIV-like enzymes. As a direct result, for example, the above-mentioned insulinotropic peptides are broken down to a lesser degree and the effectiveness of insulin is thereby increased.

The administration of unstable inhibitors of DPIV *per se* has disadvantages since they are degraded very rapidly *in vivo* and thus an even distribution of the inhibitors, especially in the human body, is impossible. In particular, upon oral administration such inhibitors are so unstable that they have virtually no activity at all. Accordingly, stable inhibitors have hitherto been used especially in the treatment of diabetes mellitus.

The present invention uses the concept to stabilize unstable inhibitors by masking them in prodrug form.

The properties of the active inhibitors according to the invention can be designed in such a way that the deactivation time of the DPIV-inhibitors e.g. by intramolecular cyclisation after their release from the prodrugs, is definable.

In particular, the compounds according to the invention have the advantage that the active inhibitors of DPIV and DPIV-like enzymes are released according to individual patients' needs.

When a compound according to the invention interacts with a DPIV molecule or a aminopeptidase N molecule, it is cleaved by these enzymes and the active inhibitor is released. The active inhibitor will inhibit DPIV and/or DPIV-like enzymes so that DPIV itself cannot cleave any further compounds for a defined time. The remaining

compounds are not degraded during this defined time and thus, constitute an inhibitor reservoir until the concentration of DPIV molecules or aminopeptidase N molecules rises again or active inhibitor molecules are eliminated or inactivated.

The invention has the further advantage that each organism will release exactly that amount of active inhibitor that is necessary to inhibit that amount of DPIV molecules, which is present in the body of the respective organism.

The present invention accordingly relates to novel compounds of unstable inhibitors of the serine protease dipeptidyl peptidase IV or DPIV-like enzymes, which can be used in the treatment of various disorders, especially of metabolic disorders associated with diabetes mellitus.

Surprisingly such masked inhibitors are additionally considerably more effective than non-masked inhibitors: if identical amounts of non-masked DP IV-inhibitors and of compounds according to the invention are used, the compounds according to the invention produce a marked improvement in glucose tolerance in Diabetic Zucker rats.

The compounds according to the present invention, are transported through the mucosa of the small intenstine without delay, for example simultaneously with nutrient intake.

Moreover, the site of action, at which the active DPIV-inhibitors are released can also be controlled by their structure.

To summarise, it may be stated that, using the compounds of the present invention, it is possible in a completely surprising manner:

1. to achieve increased action of the inhibitors;
2. to release the active inhibitors according to the patient's needs;

3. to release the active inhibitors in a temporally controlled manner;
4. to release the active inhibitors in a site-specific manner; and
5. to provide a reservoir of DPIV-inhibitors.

As indicated above, the compounds of the present invention, and their corresponding pharmaceutically acceptable acid addition salt forms, are useful in inhibiting DPIV and DPIV – like enzyme activity. The ability of the compounds of the present invention, and their corresponding pharmaceutically acceptable acid addition salt forms to inhibit DPIV and DPIV – like enzyme activity may be demonstrated employing the DPIV activity assay for determination of the K_i-values and the IC_{50}-values *in vitro*, as described in examples 2 and 3.

The ability of the compounds of the present invention, and their corresponding pharmaceutically acceptable acid addition salt forms to inhibit DPIV *in vivo* may be demonstrated by oral or intravasal administration to Wistar rats, as described in example 6. The compounds of the present invention inhibit DPIV activity *in vivo* after both, oral and intravasal administration to Wistar rats.

DPIV is present in a wide variety of mammalian organs and tissues e.g. the intestinal brush-border (Gutschmidt S. et al., "In situ" - measurements of protein contents in the brush border region along rat jejunal villi and their correlations with four enzyme activities. Histochemistry 1981, 72 (3), 467-79), exocrine epithelia, hepatocytes, renal tubuli, endothelia, myofibroblasts (Feller A.C. et al., A monoclonal antibody detecting dipeptidylpeptidase IV in human tissue. Virchows Arch. A. Pathol. Anat. Histopathol. 1986; 409 (2):263-73), nerve cells, lateral membranes of certain surface epithelia, e.g. Fallopian tube, uterus and vesicular gland, in the luminal cytoplasm of e.g., vesicular gland epithelium, and in mucous cells of Brunner's gland (Hartel S. et al., Dipeptidyl peptidase (DPP) IV in rat organs. Comparison of immunohistochemistry and activity histochemistry. Histochemistry 1988; 89 (2): 151-61), reproductive organs, e.g. cauda epididymis

and ampulla, seminal vesicles and their secretions (Agrawal & Vanha-Perttula, Dipeptidyl peptidases in bovine reproductive organs and secretions. Int. J. Androl. 1986, 9 (6): 435-52). In human serum, two molecular forms of dipeptidyl peptidase are present (Krepela E. et al., Demonstration of two molecular forms of dipeptidyl peptidase IV in normal human serum. Physiol. Bohemoslov. 1983, 32 (6): 486-96). The serum high molecular weight form of DPIV is expressed on the surface of activated T cells (Duke-Cohan J.S. et al., Serum high molecular weight dipeptidyl peptidase IV (CD26) is similar to a novel antigen DPPT-L released from activated T cells. J. Immunol. 1996, 156 (5): 1714-21).

The compounds of the present invention, and their corresponding pharmaceutically acceptable acid addition salt forms are able to inhibit DPIV *in vivo*. In one embodiment of the present invention, all molecular forms, homologues and epitopes of DPIV from all mammalian tissues and organs, also of those, which are undiscovered yet, are intended to be embraced by the scope of this invention.

Among the rare group of proline-specific proteases, DPIV was originally believed to be the only membrane-bound enzyme specific for proline as the penultimate residue at the amino-terminus of the polypeptide chain. However, other molecules, even structurally non-homologous with the DPIV but bearing corresponding enzyme activity, have been identified recently. DPIV-like enzymes, which are identified so far, are e.g. fibroblast activation protein α, dipeptidyl peptidase IV β, dipeptidyl aminopeptidase-like protein, N-acetylated α-linked acidic dipeptidase, quiescent cell proline dipeptidase, dipeptidyl peptidase II, attractin and dipeptidyl peptidase IV related protein (DPP 8), and are described in the review article by Sedo & Malik (Sedo & Malik, Dipeptidyl peptidase IV-like molecules: homologous proteins or homologous activities? Biochimica et Biophysica Acta 2001, 36506: 1-10). Further DPIV-like enzymes are disclosed in WO 01/19866, WO 02/34900 and WO02/31134. WO 01/19866 discloses novel human dipeptidyl aminopeptidase 8 (DPP8) with structural und functional similarities to DPIV and fibroblast activation protein (FAP). WO 02/34900 discloses a novel dipeptidyl

peptidase 9 (DPP9) with significant homology to the amino acid sequences of DPIV and DPP8. WO 02/31134 discloses three DPIV-like enzymes, DPRP1, DPRP2 and DPRP3.

In another preferred embodiment of the present invention, all molecular forms, homologues and epitopes of proteins comprising DPIV-like enzyme activity, from all mammalian tissues and organs, also of those, which are undiscovered yet, are intended to be embraced by the scope of this invention.

The ability of the compounds of the present invention, and their corresponding pharmaceutically acceptable acid addition salt forms to inhibit DPIV-like enzymes may be demonstrated employing an enzyme activity assay for determination of the K_i-values *in vitro* as described in example 4.

In another embodiment, the compounds of the present invention, and their corresponding pharmaceutically acceptable acid addition salt forms have only low, if no inhibitory activity against non-DPIV and non-DPIV – like proline specific enzymes. See therefore example 5.

In view of their ability to inhibit DPIV and DPIV – like enzyme activity, the compounds of the present invention, and their corresponding pharmaceutically acceptable acid addition salt forms, are useful in treating conditions mediated by said enzyme activities. Based on the findings described in the examples of the present invention and in the literature, it can be shown that the compounds disclosed herein are useful in the treatment of conditions such as immune, autoimmune disorders or central nervous system disorders, selected from the group consisting of strokes, tumors, ischemia, Parkinson's disease, and migraines.

In a more preferred embodiment of this invention, the compounds of the present invention and their corresponding pharmaceutically acceptable acid addition salt forms, improve glucose tolerance by lowering elevated blood glucose levels in

response to an oral glucose challenge and, therefore, are useful in treating non-insulin-dependent diabetes mellitus. The ability of the compounds of the present invention, and their corresponding pharmaceutically acceptable acid addition salt forms, to improve glucose tolerance in response to an oral glucose challenge, may be measured in diabetic Zucker rats. The method is described in example 7.

The present invention therefore provides a method of preventing or treating a condition mediated by modulation of the DPIV or DPIV – like enzyme activity in a subject in need thereof which comprises administering any of the compounds of the present invention or pharmaceutical compositions thereof in a quantity and dosing regimen therapeutically effective to treat the condition. Additionally, the present invention includes the use of the compounds of this invention, and their corresponding pharmaceutically acceptable acid addition salt forms, for the preparation of a medicament for the prevention or treatment of a condition mediated by modulation of the DPIV activity in a subject. The compound may be administered to a patient by any conventional route of administration, including, but not limited to, intravenous, oral, subcutaneous, intramuscular, intradermal, parenteral and combinations thereof.

In a further preferred form of implementation, the invention relates to pharmaceutical compositions, that is to say, medicaments, that contain at least one compound of the invention or salts thereof, optionally in combination with one or more pharmaceutically acceptable carriers and/or solvents.

The pharmaceutical compositions may, for example, be in the form of parenteral or enteral formulations and contain appropriate carriers, or they may be in the form of oral formulations that may contain appropriate carriers suitable for oral administration. Preferably, they are in the form of oral formulations.

The pharmaceutical compositions may additionally contain one or more

hypoglycaemically active ingredients which may be active ingredients that are known *per se*.

The effectors of DP IV and DPIV-like enzymes administered according to the invention may be employed in pharmaceutically administrable formulations or formulation complexes as inhibitors or in combination with inhibitors, substrates, pseudosubstrates, inhibitors of DP IV expression, binding proteins or antibodies of those enzyme proteins that reduce the DP IV and DPIV-like protein concentration in mammals. The compounds of the invention make it possible to adjust treatment individually to patients and diseases, it being possible, in particular, to avoid individual intolerances, allergies and side-effects.

The compounds also exhibit differing degrees of activity as a function of time. The doctor providing treatment is thereby given the opportunity to respond differently to the individual situation of patients: he is able to adjust precisely, on the one hand, the speed of the onset of action and, on the other hand, the duration of action and especially the intensity of action.

The method according to the invention represents especially a new approach to the reduction of raised blood glucose concentration in the serum of mammals. It is simple, susceptible of commercial application and suitable for use in the treatment of especially diseases that are based on above-average blood glucose values, on neurodegenerative diseases or on high blood pressure, in mammals and especially in human medicine.

The compounds are administered, for example, in the form of pharmaceutical preparations that contain the active ingredient in combination with customary additives like diluents, excipients and/or carriers known from the prior art. For example, they are administered parenterally (for example *i.v.* in physiological saline solution) or enterally (for example orally, formulated with customary carriers, such as, for example, glucose).

Depending upon their endogenous stability and their bioavailability, one or more doses of the compounds can be given per day in order to achieve the desired normalisation of the blood glucose values. For example, such a dosage range in humans may be in the range of from 0.01 mg to 250.0 mg per day, preferably in the range of from 0.01 to 100 mg of compound per kilogram of body weight.

It has been found that by administering effectors of dipeptidyl peptidase IV and DPIV-like enzyme activities in the blood of a mammal, owing to the associated temporary reduction in activity, the endogenous (or additionally exogenously administered) insulinotropic peptides Gastric Inhibitory Polypeptide 1-42 (GIP_{1-42}) and Glucagon-Like Peptide Amide-1 7-36 ($GLP\text{-}1_{7-36}$) (or other $GLP\text{-}1_{7-37}$ or analogues thereof) are, as a consequence, broken down to a lesser extent by DP IV and DP IV-like enzymes and hence the reduction in the concentration of those peptide hormones and their analogues is reduced or delayed. The increased stability of the (endogenous or exogenously supplied) incretins or their analogues, which is achieved owing to the action of DP IV effectors and which results in their being available in greater quantities for insulinotropic stimulation of the incretin receptors of the Langerhans cells in the pancreas, alters *inter alia* the effectiveness of the body's own insulin, which brings with it a stimulation of the carbohydrate metabolism of the subject treated.

As a result, the blood sugar level falls below the glucose concentration characteristic of hyperglycaemia in the serum of the subject treated. Accordingly, it is possible to prevent or alleviate metabolic abnormalities, such as impaired glucose tolerance, glucosuria, hyperlipidaemia, metabolic acidosis, diabetes mellitus, diabetic neuropathy and nephropathy and sequelae caused by diabetes mellitus in mammals, metabolism-related hypertension and cardiovascular sequelae caused by hypertension in mammals, skin diseases and diseases of the mucosae, autoimmune diseases, high blood pressure and inflammatory conditions, and psychosomatic, neuropsychiatric and depressive illnesses, such as anxiety,

depression, sleep disorders, chronic fatigue, schizophrenia, epilepsy, nutritional disorders, spasm and chronic pain.

To enhance the blood-sugar-reducing action of various antidiabetics, combinations of various orally active antidiabetics are often used. Since the antihyperglycaemic action of the compounds of the invention operates independently of other known orally administrable antidiabetics, the active ingredients of the invention are analogously suitable for use in combination therapies, in an appropriate galenical form, for achieving the desired normoglycaemic effect.

The compounds used according to the invention can accordingly be converted in a manner known *per se* into conventional formulations, such as, for example, tablets, capsules, dragées, pills, suppositories, granules, aerosols, syrups, liquid, solid and cream-like emulsions and suspensions and solutions, using inert, non-toxic, pharmaceutically suitable carriers and additives or solvents. In each of those formulations, the therapeutically effective compounds are preferably present in a concentration of approximately from 0.1 to 80 % by weight, preferably from 1 to 50 % by weight, of the total mixture, that is to say, in amounts sufficient for the mentioned dosage latitude to be obtained.

The good absorption of the compounds used according to the invention by the mucosae of the gastrointestinal tract makes it possible for many galenical preparations to be used:

The substances can be used as medicaments in the form of dragées, capsules, bitable capsules, tablets, drops, syrups or also as suppositories or as nasal sprays.

The formulations are prepared, for example, by extending the active ingredient with solvents and/or carriers, optionally with the use of emulsifiers and/or dispersants, it being possible, for example, in the case where water is used as diluent, for organic solvents to be optionally used as auxiliary solvents.

There may be mentioned as examples of excipients: water, non-toxic organic solvents, such as paraffins (for example natural oil fractions), vegetable oils (for example rapeseed oil, groundnut oil, sesame oil), alcohols (for example ethyl alcohol, glycerol), glycols (for example propylene glycol, polyethylene glycol); solid carriers, such as, for example, natural powdered minerals (for example highly disperse silica, silicates), sugars (for example raw sugar, lactose and dextrose); emulsifiers, such as non-ionic and anionic emulsifiers (for example polyoxyethylene fatty acid esters, polyoxyethylene fatty alcohol ethers, alkylsulphonates and arylsulphonates), dispersants (for example lignin, sulphite liquors, methylcellulose, starch and polyvinylpyrrolidone) and lubricants (for example magnesium stearate, talcum, stearic acid and sodium lauryl sulphate) and optionally flavourings.

Administration is carried out in the usual manner, preferably enterally or parenterally, especially orally. In the case of enteral administration, tablets may contain in addition to the mentioned carriers further additives such as sodium citrate, calcium carbonate and calcium phosphate, together with various additives, such as starch, preferably potato starch, gelatin and the like. Furthermore, lubricants, such as magnesium stearate, sodium lauryl sulphate and talcum, can be used concomitantly for tabletting. In the case of aqueous suspensions and/or elixirs intended for oral administration, various taste correctives or colourings can be added to the active ingredients in addition to the above-mentioned excipients.

In the case of parenteral administration, solutions of the active ingredients using suitable liquid carriers can be employed. In general, it has been found advantageous to administer, in the case of intravenous administration, amounts of approximately from 0.01 to 2.0 mg/kg, preferably approximately from 0.01 to 1.0 mg/kg, of body weight per day to obtain effective results and, in the case of enteral administration, the dosage is approximately from 0.01 to 2 mg/kg, preferably approximately from 0.01 to 1 mg/kg, of body weight per day.

It may nevertheless be necessary in some cases to deviate from the stated amounts, depending upon the body weight of the experimental animal or the patient or upon the type of administration route, but also on the basis of the species of animal and its individual response to the medicament or the interval at which administration is carried out. Accordingly, it may be sufficient in some cases to use less than the above-mentioned minimum amount, while, in other cases, the mentioned upper limit will have to be exceeded. In cases where relatively large amounts are being administered, it may be advisable to divide those amounts into several single doses over the day. For administration in human medicine, the same dosage latitude is provided. The above remarks apply analogously in that case.

Examples of pharmaceutical formulations

1. Capsules containing 100 mg of a compound of the invention per capsule:

For approximately 10,000 capsules a solution of the following composition is prepared:

compound of the invention	1.0 kg
glycerol	0.5 kg
polyethylene glycol	3.0 kg
water	0.5 kg
	5.0 kg

The solution is introduced into soft gelatin capsules in a manner known *per se*. The capsules are suitable for chewing or swallowing.

2. Tablets or coated tables or dragées containing 100 mg of a compound of the invention:

The following amounts refer to the preparation of 100,000 tablets:

compound of the invention,		
finely ground		10.0 kg
glucose		4.35 kg
lactose		4.35 kg
starch		4.50 kg
cellulose, finely ground		4.50 kg

The above constituents are mixed and then provided with a solution prepared from

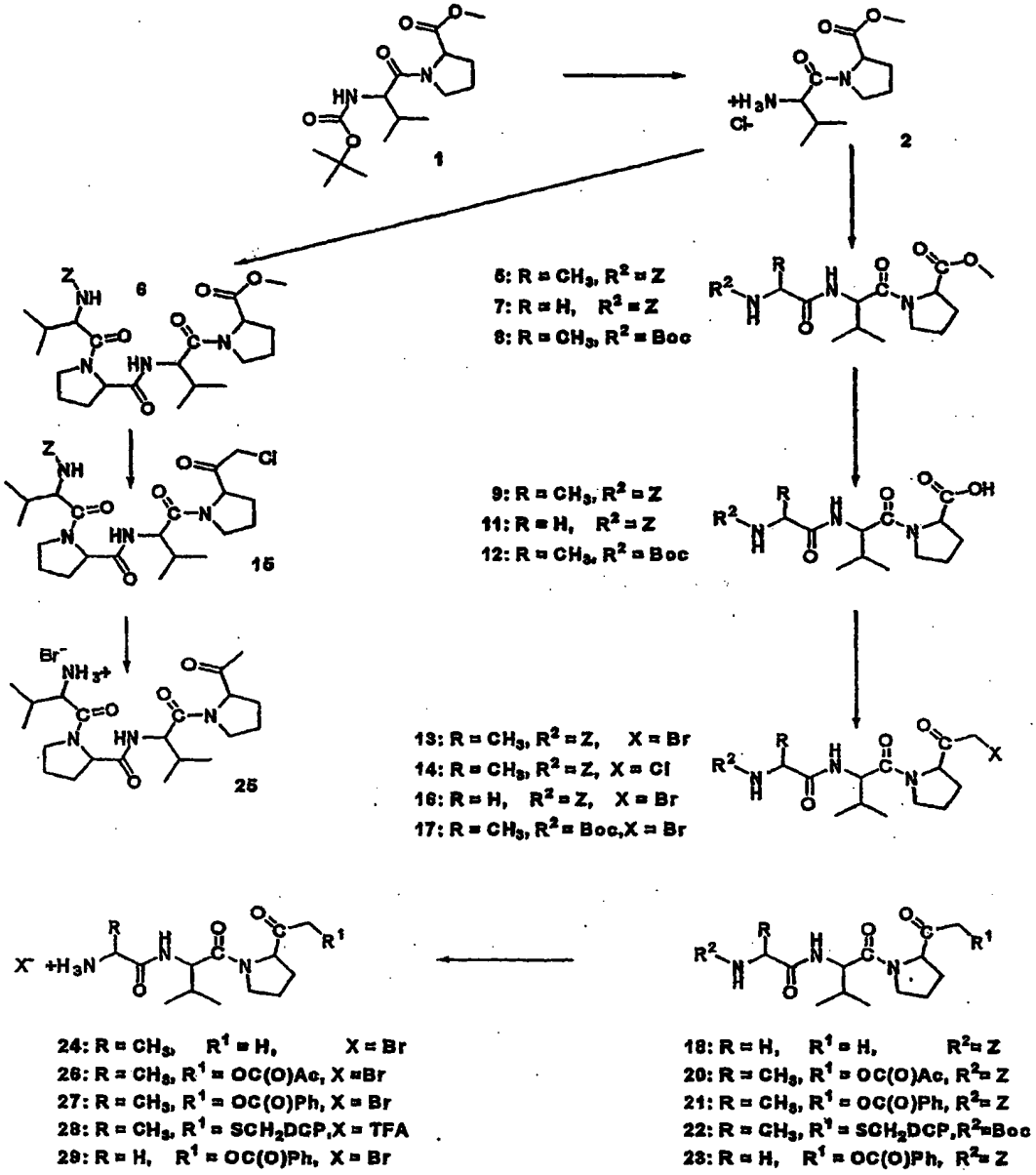
polyvinylpyrrolidone		2.0 kg
polysorbate		0.1 kg
and water	approx.	5.0 kg

and granulated in a manner known *per se* by grating the moist mass and, after the addition of 0.2 kg of magnesium stearate, drying it. The finished tablet mixture of 30.0 kg is processed to form convex tablets weighing 300 mg. The tablets can be coated or sugar-coated in a manner known *per se*.

Examples of the Invention

Example 4: Synthesis of peptidylketones

Scheme 1


1
2
6
15
25
5: R = CH3, R2 = Z
7: R = H, R2 = Z
8: R = CH3, R2 = Boc
9: R = CH3, R2 = Z
11: R = H, R2 = Z
12: R = CH3, R2 = Boc
13: R = CH3, R2 = Z, X = Br
14: R = CH3, R2 = Z, X = Cl
16: R = H, R2 = Z, X = Br
17: R = CH3, R2 = Boc, X = Br
24: R = CH3, R1 = H, X = Br
26: R = CH3, R1 = OC(O)Ac, X = Br
27: R = CH3, R1 = OC(O)Ph, X = Br
28: R = CH3, R1 = SCH2DCP, X = TFA
29: R = H, R1 = OC(O)Ph, X = Br
18: R = H, R1 = H, R2 = Z
20: R = CH3, R1 = OC(O)Ac, R2 = Z
21: R = CH3, R1 = OC(O)Ph, R2 = Z
22: R = CH3, R1 = SCH2DCP, R2 = Boc
23: R = H, R1 = OC(O)Ph, R2 = Z

H-Val-Pro-OMe*HCl *2*

Boc-Val-OH (3.00g, 13.8mmol) was dissolved in 10ml of dry THF and cooled down to –15°C. To the mixture CAIBE (1.80ml, 13.8mmol) and NMM (1.52ml, 13.8mmol) where added and the solution was stirred until the formation of the mixed anhydride was complete. Then the mixture was brought to –10°C and NMM (1.52ml, 13.8mmol) was added followed by H-Pro-OMe*HCl (2.29g, 13.8mmol). The mixture was allowed to reach r.t. and left overnight.

After removing the solvent and the usual workup the resulting ester *1* was taken without further characterisation.
1 was dissolved in HCl/HOAc (5ml, 6N) and left at 0°C until the removal of the Boc-group was complete. Then the solvent was removed and the resulting oil was treated with diethylether to give a white solid *2*.
Yield: 2.5g, 80%

Z-Val-Pro-OMe *3*

Z-Val-OH (3.5g, 13.9mmol) and H-Pro-OMe*HCl (2.14g, 13.9mmol) where treated in the same manner as above for *1*, to give *3* as a white solid.
Yield: 3.76g, 80%

Z-Val-Pro-OH *4*

3 (3.76g, 10.4mmol) was dissolved in 30 ml of water/acteone (1/5 v/v) and 11.4ml NaOH (1N) where added. After completion of the reaction the organic solvent was removed by evaporation and the resulting solution was diluted by 15ml $NaHCO_3$ solution (saturated). Then the mixture was extracted three times by 10ml of acetic acid ethyl ester. After that the solution was brought to pH2 by adding HCl (15% in water). The resulting mixture was extracted three times by 30ml of acetic acid ethyl ester. The organic layer was separated and washed three times with brine, dried (Na_2SO_4) and evaporated.

Yield: 3.25g, 90%,

Z-Ala-Val-Pro-OMe *5*

Z-Ala-OH (3.5g, 15.7mmol) and ***2*** (4.18g, 15.7mmol) where treated in the same manner as above for ***1***, to give ***3*** as a white solid.
Yield: 4.2g, 64%

Z-Val-Pro-Val-Pro-OMe *6*

4 (3.76g, 10.08mmol)and ***2*** (2.19g 10.08mmol) where treated in the same manner as above for ***1***, to give ***6*** as a white solid.
Yield: 4.21g, 70%

Z-Gly-Val-Pro-OMe *7*

Z-Gly-OH (1.55g 7.45mmol) and ***2*** (1.51g, 7.45mmol) where treated in the same manner as above for ***1***, to give ***7*** as an oil.
Yield: 2.99 g, 96%

Boc-Ala-Val-Pro-OMe *8*

Boc Ala (1.29g 6,80mmol) and ***2*** (1.80g 6,80mmol) where treated in the same manner as above for ***1***, to give ***8*** as a white solid.
Yield: 2.24 g, 83,1%

Z-Ala-Val-Pro-OH *9*

5 (4.15g, 9.6mmol) was treated in the same manner as above for ***4***, to give ***7*** as a white solid.
Yield: 3.5g ,87%

Z-Val-Pro-Val-Pro-OH *10*

6 (4.21g, 7.5mmol) was treated in the same manner as above for *4*, to give *7* as a white solid.
Yield: 3.89g ,95%

Z-Gly-Val-Pro-OH *11*

7 (2.99g, 7.15mmol) was treated in the same manner as above for *4*, to give *7* as a white solid.
Yield: 1.88g, 65%

Boc-Ala-Val-Pro-OH *12*

8 (1g 2,50mmol) was treated in the same manner as above for *4*, to give *7* as a white solid.
yield: 0,88 g, 89,1%

Z-Ala-Val-Pro-CH_2-Br *13*

9 (2.00g, 4.76mmol) was dissolved in 15ml of dry THF and converted into a mixed anhydride (see compound *1*) using CAIBE (0.623ml, 4.76mmol) and NMM (0.525 ml, 4.76mmol). The precipitate formed was filtered off and cooled down to –15°C. Then diazomethane (23.8mmol in 30ml ether) was dropped into the solution under an argon atmosphere. After leaving the mixture for 1h at 0°C 1.27ml of HBr (33% in AcOH) where added and the solution was stirred for 30min at r.t.. After that 70 ml of ether where added and the mixture was washed 20 ml of water. The organic layer was separated and dried (Na_2SO_4) and evaporated.
Yield (crude): 1.8g , 80%

Z-Ala-Val-Pro-CH_2-Cl *14*

9 (1.02g, 2.43mmol) was treated as described for ***13*** using CAIBE(0.315ml, 2.43mmol), NMM(0.267ml, 2.43mmol), diazomethane (12.2mmol in 16ml ether) and 5ml of HCl in dioxane (7.6M).
Yield (crude): 1g 91%

Z-Val-Pro-Val-Pro-CH_2-Cl *15*

10 (1.1g, 2.01mmol) was treated as described for ***13*** using CAIBE(0.263ml, 2.01mmol), NMM(0.223ml, 2.02mmol), diazomethane (10mmol in 13.3ml ether) and 5ml of HCl in dioxane (7.6M).
Yield (crude): 1.1g, 95%

Z-Gly-Val-Pro-CH_2-Br *16*

11 (2.04g, 5.05mmol) was treated as described for ***13*** using CAIBE(0.656ml, 5.05mmol), NMM(0.556ml, 5.05mmol), diazomethane (10mmol in 13.3ml ether) and 5ml of HCl in dioxane (7.6M).
Yield (crude): 2.10g, 90.4%

Boc-Ala-Val-Pro-CH_2Br *17*

12: (0.88g, 2.28mmol) was treated as described for ***13***, using CAIBE (2.28mmol, 0.37ml), NMM (2.28mmol, 0,31 ml), diazomethane (14.3mmol in 15ml ether) HBr/glacial acetic acid (33%): 4.24mmol, 1.04 ml
Yield:0.88g, 83.4%

Z-protected methylketones

Z-Ala-Val-Pro-CH_3 *18*

14 (1g, 2.21mmol) was dissolved in 5.30ml of warm acidic acid and 1.33 of zink-powder was added portion wise to the stirred solution. After 24h the remaining solid was filtered off and the filtrate was evaporated. The remaining oil was taken up in ethylacetate and washed twice with $NaHCO_3$ and brine. The organic layer was then dried and evaporated and purified by column chromatography using a heptane/chloroform/methanol-gradient.
Yield: 0.230g (24.8%)

Z-Val-Pro-Val-Pro-CH_3 *19*

15 (1.1g, 1.91mmol) was treated as described for *18* using acidic acid (5.3ml) and zink (1.31g).
Yield: 0.190g, 16%

N-protected acyloxymethylene ketones

The acid (2eq) was dissolved in DMF and an equimolar amount of KF was added. The suspension was allowed to stir at r.t. for 1h. Then the brommethylene (1eq) component was added and the solution was allowed to stir overnight.
After that the solvent was removed under vacuum and the resulting oil was dissolved in chloroform and washed with brine. Then the organic layer was separated dried (Na_2SO_4) and the solvent was removed. The product was purified by column chromatography using silica gel and heptane/chloroform.

Z-Ala-Val-Pro-CH_2O-C(O)-CH_3 *20*

Acetic acid (230µl, 4.02mmol), KF (0.234g, 4.02mmol), *13* (1.00g, 2.01mmol)
Yield: 0.351g, 36%

Z-Ala-Val-Pro-CH_2O-C(O)-Ph *21*

Benzoic acid (0.275g, 2.25mmol), KF (0.131mg, 2.25mmol), ***13*** (0.56g. 1.13mmol)
Yield: 0.34g, 56%

Boc-Ala-Val-Pro-CH_2-S-CH_2-Dichlorphenyl *22*

Dichlorbenzylmercaptane (0.30ml, 2.09 mmol), KF (0.250g, 4.19mmol), ***17*** (0.88g. 1.9mmol)
Yield: 0,56 g, 51%

Z-Gly-Val-Pro-CH_2O-C(O)-Ph *23*

Benzoic acid (1.19g, 9.78mmol), KF (0.568g, 9.78mmol), ***16*** (2.35g. 4.89mmol)
Yield: 0.89g, 34.8%

Deprotection

Method A
The Z-protected compound was dissolved in HBr/AcOH and stirred. When the reaction was complete ether was added, the white precipitate formed was filtered off and dried.
Method B:
The Boc-protected compound was dissolved in TFA and stirred. When the reaction was complete ether was added, the white precipitate formed was filtered off and dried.

H-Ala-Val-Pro-CH_3*HBr *24*

Method A
18 (0.230g, 0.54mmol)
Yield: 0.124g, 80%

H-Val-Pro-Val-Pro-CH_3*HBr *25*

Method A

19 (0.190g, 0.20mmol)

Yield: 0.114g, 82.3%

H-Ala-Val-Pro-CH_2O-C(O)CH_3*HBr *26*

Method A

20 (0.351g, 0.73mmol)

Yield: 0.252g, 98%

H-Ala-Val-Pro-CH_2O-C(O)Ph*HBr *27*

Method A

21 (0.34g, 0.63mmol)

Yield: 0,251g, 99%

H-Ala-Val-Pro-CH_2-S-CH_2-Dichlorphenyl*TFA *28*

Method B

22 (0.56g, 0.97 mmol)

Yield: 0.027g, 5%

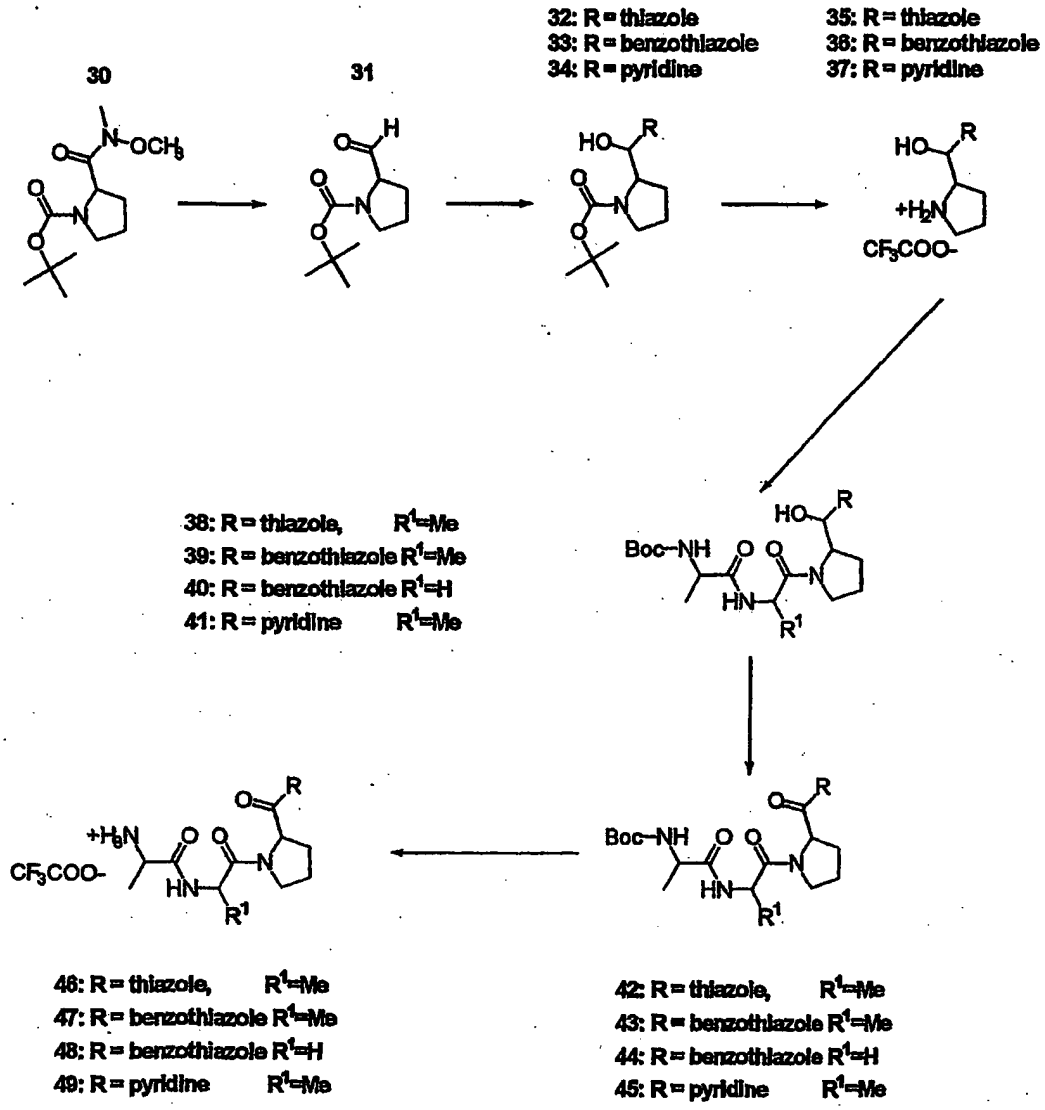
H-Gly-Val-Pro-CH_2O-C(O)Ph*HBr *29*

Method A

23 (0.156g, 0.26mmol)

Yield: 0.115g, 99%

Scheme 2



Boc-Pro-N(Me)OMe *30*

Boc-Prolin (2.00g, 9.29 mmol) and N,O-Dimethylhydroxylaminhydrochloride (0.91g 9.29 mmol) where treated as described for ***1*** using NMM (2ml, 18.4) and CAIBE (1.47ml, 9.29 mmol)

Yield: 2.1 g, 87.5 %)

Boc-Prolinal *31*

10 mmol of ***30*** where dissolved in 20ml of absolute ether at 0°C. 12,5mmol lithiumalanat where added. After 7 min of stirring 10ml of a saturated $KHSO_4$ solution where added drop wise. Then the mixture was diluted by adding 50 ml of ether and the organic layer was separated. This was washed by 1N HCl, water, saturated $NaHCO_3$– solution, brine and dried

30 (1.43g, 5.54mmol), $LiAlH_4$ (0.26g, 6.92mmol)

Yield: 0.78g, 70.8%

2-(Heterocyclo-hydroxymethyl-N-Boc-(2S)-pyrrolidines

1.1 eq of the heterocycle where dissolved in 5 ml of dry THF under argon atmosphere and brought to –65°C. 1.1eq of n-Butyllithium (1,6 M in Hexan) where added and the solution was stirred at –65°C for 1h. 1eq of ***31***, dissolved in 2ml dry THF, was dropped into the stirred solution and the mixture was stirred at –65°C for 2h. After that 2ml of water where added and the solution was extracted three times using methylene chloride. The organic layer was separated, dried and evaporated.

2-[([1,3]-Thiazol-2-yl)hydroxymethyl]-1-N-(tert-butoxycarbonyl)-(2S)-pyrrolidine *32*

31 (1.0g, 5.02mmol), thiazole (0.39ml, 5.52mmol), n-BuLi (1,6 M) (3.45ml 5.52mmol)

Yield: 1.02g, 71.0%

2-[Benzothiazol-2-yl)hydroxymethyl]-1-N-(tert-butoxycarbonyl)-(2S)-pyrrolidine *33*

31 (1.0g, 5.02mmol), benzothiazole (0.6ml, 5.52mmol), n-BuLi (1,6M) (3.45ml 5.52mmol)

Yield: 1.73g, 78.0%

2-[(Pyridin-2-yl)hydroxymethyl]-1-N-(tert-butoxycarbonyl)-(2S)-pyrrolidine *34*

31: (1.3g, 6.54mmol), 2-brompyridine (0.70ml, 7.19mmol), n-BuLi (1.6M) (4.5ml, 7.19mmol)

Yield: 1.68g, 92.2%

2-(Heterocyclo-hydroxymethyl-(2S)-pyrrolidines

32, ***33*** and ***34*** where treated as described for ***2***.

2-[([1,3]-Thiazol-2-yl)hydroxymethyl]-(2S)-pyrrolidin hydrochlorid *35*

32 (0.46g, 1.62mmol)

Yield: 0.34g, 94.9%

2-[(Benzothiazol-2-yl)hydroxymethyl]-(2S)-pyrrolidine hydrochloride *36*

33 (0.6g, 1.79mmol)

Yield: 0.436g, 90%

2-[(Pyridin-2-yl)hydroxymethyl]-(2S)-pyrrolidine hydrochloride *37*

34 (0.95g, 3.41mmol)

Yield: 0.71g, 96.8%

N protected 2-(Heterocyclo-hydroxymethyl-N-Peptidyl-(2S)-pyrrolidines

1eq of a Boc-Ala-Val-OH or Boc-Ala-Gly-OH and 1eq of N-hydroxysuccinimide where dissolved in dioxane. At 0°C 1eq of dicyclohexylcarbodiimide where added and the solution was stirred for 2h. After stirring overnight at r.t. the precipitate was removed. The organic phase was washed with a saturated solution of $NaHCO_3$ and brine. After drying the solvent was removed.

The active ester was dissolved with 1eq of ***35*** in dry THF and brought to 0°C. 1eq of triethylamin was added and stirred for 2h at 0°C. The solvent was removed and

the resulting oil was dissolved in ethyl acetate. After washing with 1N HCl, water, a saturated solution of $NaHCO_3$ and brine the solvent was removed after drying.
The mixture was purified by column chromatography using a heptane/chloroforme gradient

2-[([1,3]-Thiazol-2-yl)hydroxymethyl]-1-{N-[N-(tertbutyloxycarbonyl) (L)-Alanyl]-(L)-Valinyl}-(2S)-pyrrolidine ***38***

35 (0.44g, 1.54mmol), N-hydroxysuccinimid (0.17g, 1.54mmol), DCC (0.32g, 1.54mmol), Boc-Ala-Val-OH (0.34g, 1.54mmol), TEA (0.22ml, 1.54mmol)
Yield: 0.3g, 42.9%

1eq of Boc-Ala-Val-OH or Boc-Ala-Gly-OH and 0.9eq of ***36*** or ***37***, 1.1eq of HOBt, and 1.1eq of WSCD where dissolved in dry ACN. After addition of 0.9eq of TEA the mixture was stirred overnight. The solvent was removed and the remaining oil was dissolved in ethyl acetate. The solution was washed with brine and dried.

2-[(Benzothiazol-2-yl)hydroxymethyl]-1-{N-[N-(tertbutyloxycarbonyl)-(L)-Alanyl]-(L)-Valinyl}-(2S)-pyrrolidine ***39***

36 (0.43g, 1.23mmol), HOBt (0.183g, 1.35mmol), WSCD (0.259g, 1.35mmol), Boc-Ala-Val-OH (0.35g, 1.23mmol), TEA (0.172ml, 1.54mmol)
Yield: 0.3g, 42.9%

2-[(Benzothiazol-2-yl)hydroxymethyl]-1-{N-[N-(tertbutyloxycarbonyl) (L)-Alanyl]-glycyl}-(2S)-pyrrolidin ***40***

36 (0.43g, 1.23mmol), HOBt (0.183g, 1.35mmol), WSCD (0.259g, 1.35mmol), Boc-Ala-Val-OH (0.303g, 1.23mmol), TEA (0.172ml, 1.54mmol)
Yield: 0.41g, 72%

2-[(pyridin-2-yl)hydroxymethyl]-1-{N-[N-(tertbutyloxycarbonyl) (L)-Alanyl]-(L)-Valinyl}-(2S)-pyrrolidin *41*

37 (0.15g, 0.52mmol) Boc-AlaVal-OH (0.1g, 0.47mmol); HOBT (0.08g, 0.57mmol), WSCD (0.11g, 0.57mmol), TEA (0.07ml, 0.47mmol)
Yield: 0.22g, 94.9%

N protected 2-(Heterocyclo-carbonyl-N-Peptidyl-(2S)-pyrrolidines

1.8eq of oxaylchlorid where dissolved in 5ml of dry dichlormethane and brought to –78°C under argon atmosphere. A solution of 2.5eq of DMSO in 2ml dichlormethane was added and kept for 20min at–78°C. 1eq of ***38***, ***39***, ***40*** or ***41*** where dissolved in 5ml dichlormethane and added drop wise. The mixture was stirred for 20min at –78°C. After that 4eq TEA was added and the mixture was brought to r.t.. 30ml of a mixture of hexane/ethyl acetate (2/1, v/v) and 10ml of a 2% HCl (m/V) where added. The organic layer was separated dried and the solvent was removed.
The mixture was purified by column chromatography using a heptane/chloroforme gradient

2-[([1,3]-thiazol-2-yl)carbonyl]-1-{N-[N-(tertbutyloxycarbonyl) (L)-Alanyl]-(L)-Valinyl}-(2S)-pyrrolidin *42*

38: (0.15g, 0.33mmol), oxalylchloride (0.05ml, 0.59mmol), DMSO (0.06ml, 0.82mmol), TEA (0.12ml, 1.32mmol)
Yield: 0.13g, 89%

2-[(Benzothiazol-2-yl)carbonyl]-1-{N-[N-(tertbutyloxycarbonyl)-(L)-Alanyl]-(L)-Valinyl}-(2S)-pyrrolidine *43*

39: (0.72g, 0.14mmol), oxalylchloride (0.221ml, 2.57mmol), DMSO (2.53ml, 3.57mmol), TEA (0.80ml, 5.71 mmol)

Yield: 0.049g, 70%

2-[(Benzothiazol-2-yl)carbonyl]-1-{N-[N-(tertbutyloxycarbonyl)-(L)-Alanyl]-glycyl}-(2S)-pyrrolidine *44*

40: (0.62g, 0.134mmol), oxalylchloride (0.207ml, 2.41 mmol), DMSO (2.37ml, 3.35mmol), TEA (0.75ml, 5.35mmol)
Yield: 0.38g, 62%

2-[(Pyridin-2-yl)carbonyl]-1-{N-[N-(tertbutyloxycarbonyl) (L)-Alanyl]-(L)-Valinyl}-(2S)-pyrrolidine *45*

41 (0.29g, 0.64mmol), oxalylchloride (0.10ml, 1.15mmol), DMSO (0.11ml, 1.59mmol), TEA (0.36ml, 2,55mmol)
Yield: 0.14g, 49,2%

2-(Heterocyclo-carbonyl-N-Peptidyl-(2S)-pyrrolidines

42, ***43***, ***44***, ***45*** where treated as described under deprotection method B.

2-[([1,3]-Tthiazol-2-yl)carbonyl]-1-N-[N-{(L)-Alanyl}-(L)-Valinyl]-(2S)-pyrrolidin triflouracetat *46*

42 (0.13g, 0.29mmol)
Yield: 0.04g, 30.1%)

2-[(Benzothiazolethiazol-2-yl)carbonyl]-1-N-[N-{(L)-Alanyl}-(L)-Valinyl]-(2S)-pyrrolidin trifluoracetat *47*

43 (0.49g, 0.97mmol)
Yield: 0.24g, 60.3%)

2-[(Benzothiazolethiazol-2-yl)carbonyl]-1-N-[N-{(L)-Alanyl}-Glycyl]-(2S)-pyrrolidine trifluoracetat *48*

44 (0.38g, 0.82mmol)
Yield: 0.187g, 60.1%

2-[(Pyridin-2-yl)carbonyl]-1-N-[N-{(L)-Alanyl}-(L)-Valinyl]-(2S)-pyrrolidine triflouracetat *49*

45 (0.14g, 0.31mmol)
yield: 0.054g, 37.7%

From the compounds of the present invention biological efficacy data were investigated. The results are described and discussed in the further examples. In particular, these compounds are:

Cpd. no.	Short name	Full name
24	H-Ala-Val-Pro-Me*HBr	*2-Methylcarbonyl-1-N-[(L)-Alanyl-(L)-Valinyl]-(2S)-pyrrolidine hydrobromide*
25	H-Val-Pro-Val-Pro-Me*HBr	*2-Methyl)carbonyl-1-N-[(L)-Valinyl-(L)-Prolyl-(L)-Valinyl]-(2S)-pyrrolidine hydrobromide*
26	H-Ala-Val-Pro-CH_2O-CO-CH_3*HBr	*2-[(Acetyl-oxy-methyl)carbonyl]-1-N-[(L)-Alanyl-(L)-Valinyl]-(2S)-pyrrolidine hydrobromide*
27	H-Ala-Val-Pro-CO-CH_2O-CO-Ph*HBr	*2-[Benzoyl-oxy-methyl)carbonyl]-1-N-[{(L)-Alanyl}-(L)-Valinyl]-(2S)-pyrrolidine hydrobromide*
28	H-Ala-Val-Pro-CO-CH_2-S-CH_2-Dichlorphenyl*TFA	*2-[[(2,6-Dichlorbenzyl)thiomethyl]carbonyl]-1-N-[{(L)-Alanyl}-(L)-Valinyl]-(2S)-pyrrolidine*
29	H-Gly-Val-Pro-CO-CH_2O-CO-Ph*HBr	*2-[Benzoy-loxy-methyl)carbonyl]-1-N-[Glycyl-(L)-Valinyl]-(2S)-pyrrolidine hydrobromide*
46	H-Ala Val-Pro-CO-Thiazol*TFA	*2-[([1,3]-Thiazolethiazol-2-yl)carbonyl]-1-N-[{(L)-Alanyl}-(L)-Valinyl]-(2S)-pyrrolidine trifluoracetat*
47	H-Ala-Val-Pro-CO-	*2-[(Benzothiazolethiazol-2-yl)carbonyl]-1-N-*

	Benzothiazole*TFA	*[N-[(L)-Alanyl]-(L)-Valinyl]-(2S)-pyrrolidin trifluoracetat*
48	H-Ala-Gly-Pro-CO-Benzothiazol*TFA	*2-[(-Benzothiazolethiazol-2-yl)carbonyl]-1-N-[[(L)-Alanyl]-Glycyl]-(2S)-pyrrolidine trifluoracetat*
49	H-Ala-Val-Pro-CO-(2-Pyridine)*TFA	*2-[(Pyridin-2-yl)carbonyl]-1-N-[N-[(L)-Alanyl]-(L)-Valinyl]-(2S)-pyrrolidine trifluoracetat*

Example 2: K_i-determination

For K_i determination dipeptidyl peptidase IV from porcine kidney with a specific activity against glycylprolyl-4-nitroaniline of 37.5 U/mg and an enzyme concentration of 1.41 mg/ml in the stock solution was used.

Assay mixture:

500 µl test compound in a concentration range of $1*10^{-5}$ M – $1*10^{-11}$M were admixed with and 500 µl HEPES buffer (40 mM, pH7.6; ion strength = 0.125) and 20 µl of diluted DPIV. Release of the inhibitor from the prodrug as well the monitoring reaction (DPIV-catalyzed hydrolysis of Gly-Pro-pNA) were started by addition of a mixture of 10 µl of APN stock solution (4,9 mg/ml, Sigma, Taufkirchen, Germany) with 250 µl of the substrate (Gly-Pro-pNA, 0.05 - 4 mM). Development of yellow color due to 4-nitroaniline release were monitored at □ = 405 nm for up to 180 min using UV1 Spectrometer (ThermoSpectronic).

The K_i-values were calculated by fitting the first derivative of the time-progress curves using Graphit (v.4.0.13, Erithacus Software, Ltd, UK) and a equation for an unstable competitive inhibitor.

$$v = \frac{V_{max} * S_0}{S_0 + K_m\left(1 + \frac{I * e^{-kt}}{K_i}\right)}$$

The half-life ($t_{½}$) was calculated by plotting the enzyme activity versus reaction time.

2.1 Results - K_i values of DPIV inhibition

Compound	KI [M]	$T_{½}$ [min]
H-Ala-Val-Pro-Me*HBr	$4.76*10^{-8}$	12.4
H-Val-Pro-Val-Pro-Me*HBr	n.d.	n.d.
H-Ala-Val-Pro-CH_2O-CO-CH_3*HBr	$1.05*10^{-9}$	10.8
H-Ala-Val-Pro-CO-CH_2O-CO-Ph*HBr	$5.36*10^{-10}$	15.1
H-Gly-Val-Pro-CO-CH_2O-CO-Ph*HBr	no inhibition	n.d.
H-Ala-Val-Pro-CO-Benzothiazole*TFA	$3.73*10^{-8}$	17.0
H-Ala-Gly-Pro-CO-Benzothiazole*TFA	$1.07*10^{-7}$	5.1
H-Ala Val-Pro-CO-Thiazole*TFA	$3.32*10^{-8}$	15.1
H-Ala-Val-Pro-CO-(2-Pyridinyl)*TFA	n.d.	n.d.
H-Ala-Val-Pro-CO-CH_2-S-CH_2-Dichlorphenyl*TFA	$<1.0*10^{-7}$	n.d.

n.d. not determined

Example 3: Determination of IC_{50}-Values

100 µl inhibitor stock solution were mixed with 100 µl buffer (HEPES pH7.6) and 20 µl diluted porcine DPIV and preincubated at 30°C. Reaction was started by addition of a mixture of 50 µl substrate (Gly-Pro-pNA, final concentration 0.4 mM) and 2µl APN stock solution. Formation of the product pNA was measured at 405 nm and 30°C over 10 min using the HTS 7000Plus plate reader (Perkin Elmer) and slopes were calculated. The final inhibitor concentrations ranged between 1 mM and 30 nM. For calculation of IC50 GraFit 4.0.13 (Erithacus Software) was used.

3.1 Results – Determination of IC_{50} values

Compound	IC_{50} [M]
H-Ala-Val-Pro-Me*HBr	$5,79*10^{-7}$
H-Val-Pro-Val-Pro-Me*HBr	n.d.
H-Ala-Val-Pro-CH_2O-CO-CH_3*HBr	$1,02*10^{-8}$
H-Ala-Val-Pro-CO-CH_2O-CO-Ph*HBr	$1,79*10^{8}$
H-Gly-Val-Pro-CO-CH_2O-CO-Ph*HBr	$4,94*10^{-6}$
H-Ala-Val-Pro-CO-Benzothiazole*TFA	n.d.
H-Ala-Gly-Pro-CO-Benzothiazole*TFA	n.d.
H-Ala Val-Pro-CO-Thiazole*TFA	no inhibition

H-Ala-Val-Pro-CO-(2-Pyridinyl)*TFA	$1{,}10*10^{-3}$
H-Ala-Val-Pro-CO-CH_2-S-CH_2-Dichlorphenyl*TFA	$7{,}97*10^{-5}$

n.d. not determined

Example 4:

Inhibition Of DPIV-Like Enzymes – Dipeptidyl Peptidase II (DP II)

DP II (3.4.14.2) releases N-terminal dipeptides from oligopeptides if the N-terminus is not protonated (McDonald, J.K., Ellis, S. & Reilly, T.J., 1966, *J. Biol. Chem.*, 241, 1494-1501). Pro and Ala in P_1-position are preferred residues. The enzyme activity is described as DPIV-like activity, but DP II has an acidic pH-optimum. The enzyme used was purified from porcine kidney.

Assay:

100 µl inhibitor in an concentration range of $1*10^{-4}$ M – $5*10^{-8}$ M were admixed with 100 µl µl buffer solution (40 mM HEPES, pH7.6, 0.015% Brij, 1 mM DTT), 50 µl lysylalanylaminomethylcoumarine solution (5 mM) and 20 µl porcine DP II (250fold diluted in buffer solution). Fluorescence measurement was performed at 30°C and $\lambda_{excitation}$ = 380 nm, $\lambda_{emission}$ = 465 nm for 25 min using a plate reader (HTS7000plus, Applied Biosystems, Weiterstadt, Germany). The K_i-values were calculated using Graphit 4.0.15 (Erithacus Software, Ltd., UK).

Results:

The compound H-Ala-Val-Pro-CO-CH_2O-CO-Ph*HBr was exemplarily tested against DP II. No inhibition of DP II by H-Ala-Val-Pro-CO-CH_2O-CO-Ph*HBr was found.

Attractin

100 µl inhibitor stock solution were mixed with 100 µl buffer (HEPES pH7.6) and 20 µl diluted attractin and preincubated at 30°C. Reaction was started by addition of a

mixture of 50 µl substrate (Gly-Pro-pNA, final concentration 0.4 mM) and 2µl APN stock solution. Formation of the product pNA was measured at 405 nm and 30°C over 10 min using the HTS 7000Plus plate reader (Perkin Elmer) and slopes were calculated. The final inhibitor concentrations ranged between 1 mM and 30 nM. For calculation of IC_{50} values, GraFit 4.0.13 (Erithacus Software) was used.

Results:

The compound H-Ala-Val-Pro-Me*HBr was exemplarily tested against attractin. No inhibition of attractin by H-Ala-Val-Pro-Me*HBr was found.

Example 5: Cross Reacting Enzymes

The inhibitors were tested for their cross reacting potency against dipeptidyl peptidase I, prolyl oligopeptidase and Prolidase.

Dipeptidyl peptidase I (DP I, cathepsin C):

DP I or cathepsin C is a lysosomal cysteine protease which cleaves off dipeptides from the N-terminus of their substrates (Gutman, H.R. & Fruton, J.S., 1948, *J. Biol: Chem.*, 174, 851-858) . It is classified as a cysteine protease. The enzyme used was purchased from Qiagen (Qiagen GmbH, Hilden, Germany). In order to get a fully active enzyme, the enzyme was diluted 1000fold in MES buffer pH5,6 (40 mM MES, 4 mM DTT, 4 mM KCl, 2 mM EDTA, 0.015% Brij) and pre-incubated for 30 min at 30°C.

Assay:

50 µl solution with the test compounds in a concentration range of $1*10^{-5}$ M – $1*10^{-7}$ M were admixed with 110 µl buffer-enzyme-mixture. The assay mixture was pre-incubated at 30 °C for 15 min. After pre-incubation, 100 µl histidylseryl-βnitroaniline ($2*10^{-5}$M) was added and measurement of yellow color development due to β-nitroaniline release was performed at 30°C and $\lambda_{excitation}$ = 380 nm, $\lambda_{emission}$ =

465 nm for 10 min., using a plate reader (HTS7000 plus, Applied Biosystems, Weiterstadt, Germany).

The IC_{50}-values were calculated using Graphit 4.0.15 (Erithacus Software, Ltd., UK).

Prolidase (X-Pro dipeptidase)

Prolidase (EC 3.4.13.9) was first described by Bergmann & Fruton (Bergmann, M. & Fruton, JS, 1937, *J. Biol. Chem.* 189-202). Prolidase releases the N-terminal amino acid from Xaa-Pro dipeptides and has a pH optimum between 6 and 9.

Prolidase from porcine kidney (ICN Biomedicals, Eschwege, Germany). was solved (1mg/ml) in assay buffer (20mM $NH_4(CH_3COO)_2$, 3mM $MnCl_2$, pH 7.6). In order to get a fully active enzyme the solution was incubated for 60 min at room temperature.

Assay:

450 µl solution with the test compounds in an concentration range of $5*10^{-3}$ M – $5*10^{-7}$ M were admixed with 500 µl buffer solution (20mM $NH_4(CH_3COO)_2$, pH 7.6) and 250 µl Ile-Pro-OH (0.5mM in the assay mixture). The assay mixture was pre-incubated at 30 °C for 5 min. After pre-incubation, 75 µl Prolidase (1:10 diluted in assay buffer) were added and measurement was performed at 30°C and λ = 220 nm for 20 min using a UV/Vis photometer, UV1 (Thermo Spectronic, Cambridge, UK).

The IC_{50}-values were calculated using Graphit 4.0.15 (Erithacus Software, Ltd., UK).

Angiotensin-I converting enzyme (ACE)

Angiotensin I-converting enzyme (ACE; peptidyl-dipeptidase A) is a zinc metallopeptidase which cleaves the C-terminal dipeptide from angiotensin I to produce the potent vasopressor octapeptide angiotensin II (SkeggsL.T., Kahn, J.R. & Shumway, N.P. (1956) The preparation and function of the hypertensin-converting enzyme. J. Exp. Med. 103, 295-299.) and inactivates bradykinin by the sequential removal of two C-terminal dipeptides (YangH.Y.T., Erdös, E.G. & Levin, Y. (1970) A dipeptidyl carboxypeptidase that converts angiotensin I and inactivates bradykinin. Biochim. Biophys. Acta 214, 374-376.). In addition to these two main physiological substrates, which are involved in blood pressure regulation and water and salt metabolism, ACE cleaves C-terminal dipeptides from various oligopeptides with a free C-terminus. ACE is also able to cleave a C-terminal dipeptide amide.

Assay:

For IC_{50} determination of ACE an enzyme produced by Sigma was used (Prod.No. A-6778). The assay procedure and calculation of activity described by the manufacturer was adapted to half of the described volumes.

The IC_{50}-values were calculated using Graphit 4.0.15 (Erithacus Software, Ltd., UK).

Acylamino acid-releasing enzyme (AARE)

Acylaminoacyl-peptidase (EC 3.4.19.1) has also been referred to by the names acylpeptide hydrolase (GadeW. & Brown, J.L. (1978) Purification and partial characterization of a-N-acylpeptide hydrolase from bovine liver. J. Biol. Chem. 253, 5012-5018.; JonesW.M. & Manning, J.M. (1985) Acylpeptide hydrolase activity from erythrocytes. Biochem. Biophys. Res. Commun. 126, 933-940.; KobayashiK., Lin, L.-W., Yeadon, J.E., Klickstein, L.B. & Smith, J.A. (1989) Cloning and sequence analysis of a rat liver cDNA encoding acylpeptide hydrolase. J. Biol. Chem. 264, 8892-8899), acylamino acid-releasing enzyme (TsunasawaS., Narita, K. & Ogata, K. (1975) Purification and properties of acylamino acid-releasing enzyme from rat liver. J. Biochem. 77, 89-102.; MittaM., Asada, K., Uchimura, Y., Kimizuka, F., Kato, I., Sakiyama, F. & Tsunasawa, S. (1989) The

primary structure of porcine liver acylamino acid-releasing enzyme deduced from cDNA sequences. J. Biochem. 106, 548-551.) and acylaminoacyl peptide hydrolase (RadhakrishnaG. & Wold, F. (1989) Purification and characterization of an N-acylaminoacyl-peptide hydrolase from rabbit muscle. J. Biol. Chem. 264, 11076-11081.). Acylaminoacyl peptidase catalyzes the removal of an N-acylated amino acid from a blocked peptide: Block-Xaa↓Xbb-Xcc.... The products of the reaction are the free acyl amino acid and a peptide with a free N-terminus shortened by one amino acid. The enzyme acts on a variety of peptides with different N-terminal acyl groups, including acetyl, chloroacetyl, formyl and carbamyl (JonesW.M., Scaloni, A., Bossa, F., Popowicz, A.M., Schneewind, O. & Manning, J.M. (1991) Genetic relationship between acylpeptide hydrolase and acylase, two hydrolytic enzymes with similar binding but different catalytic specificities. Proc. Natl Acad. Sci. USA 88, 2194-2198.).

Assay:

100 µl solution with the inhibitors in an concentration range of $1*10^{-4}$ M – $5*10^{-8}$ M were admixed with 100 µl µl buffer solution (200 mM Natriumphosphat, pH 7.2) and 20 µl AARE solution. The assay mixture was pre-incubated at 30 °C for 15 min. After pre-incubation, 50 µl Acetyl-Met-AMC solution (0.54 mM) was added. Release of the AMC was measured at 30°C using a Novovostar flourescence microplate reader (BMG) and excitation/emission wavelengths of 380/460 nm.

The IC_{50}-values were calculated from the slopes of the progress curves using Graphit 4.0.15 (Erithacus Software, Ltd., UK).

5.1 Results – Determination of IC_{50} values against cross-reacting enzymes

Compound	DP I IC_{50} [M]	Prolidase IC_{50} [M]	ACE IC_{50} [M]	AARE IC_{50} [M]
H-Ala-Val-Pro-Me*HBr	no inhibition	$4.13*10^{-4}$	no inhibition	no inhibition
H-Ala-Val-Pro-CH_2O-CO-CH_3*HBr	$1.20*10^{-4}$	no inhibition	no inhibition	no inhibition
H-Ala-Val-Pro-CO-	$3.16*10^{-4}$	$4.14*10^{-4}$	no inhibition	no inhibition

CH_2O-CO-Ph*HBr				

Example 6: Determination Of DPIV Inhibiting Activity After Intravasal And Oral Administration To Wistar Rats

Animals

Male Wistar rats (Shoe: Wist(Sho)) with a body weight ranging between 250 and 350 g were purchased from Tierzucht Schönwalde (Schönwalde, Germany).

Housing conditions

Animals were single-caged under conventional conditions with controlled temperature (22±2 °C) on a 12/12 hours light/dark cycle (light on at 06:00 AM). Standard pelleted chow (ssniff® Soest, Germany) and tap water acidified with HCl were allowed ad libitum.

Catheter insertion into carotid artery

After ≥one week of adaptation at the housing conditions, catheters were implanted into the carotid artery of Wistar rats under general anaesthesia (i.p. injection of 0.25 ml/kg b.w. Rompun® [2 %], BayerVital, Germany and 0.5 ml/kg b.w. Ketamin 10, Atarost GmbH & Co., Twistringen, Germany). The animals were allowed to recover for one week. The catheters were flushed with heparin-saline (100 IU/ml) three times per week. In case of catheter dysfunction, a second catheter was inserted into the contra-lateral carotid artery of the respective rat. After one week of recovery from surgery, this animal was reintegrated into the study. In case of dysfunction of the second catheter, the animal was withdrawn from the study. A new animal was recruited and the experiments were continued in the planned sequence, beginning at least 7 days after catheter implantation.

Experimental design

Rats with intact catheter function were administered placebo (1 ml saline, 0.154 mol/l) or test compound via the oral and the intra-vasal (intra-arterial) route.

After overnight fasting, 100 µl samples of heparinised arterial blood were collected at –30, -5, and 0 min. The test substance was dissolved freshly in 1.0 ml saline (0.154 mol/l) and was administered at 0 min either orally via a feeding tube (75 mm; Fine Science Tools, Heidelberg, Germany) or via the intra-vasal route. In the case of oral administration, an additional volume of 1 ml saline was injected into the arterial catheter. In the case of intra-arterial administration, the catheter was immediately flushed with 30 µl saline and an additional 1 ml of saline was given orally via the feeding tube.

After application of placebo or the test substances, arterial blood samples were taken at 2.5, 5, 7.5, 10, 15, 20, 40, 60 and 120 min from the carotid catheter of the conscious unrestrained rats. All blood samples were collected into ice cooled Eppendorf tubes (Eppendorf-Netheler-Hinz, Hamburg, Germany) filled with 10 µl 1M sodium citrate buffer (pH 3.0) for plasma DPIV activity measurement. Eppendorf tubes were centrifuged immediately (12000 rpm for 2 min, Hettich Zentrifuge EBA 12, Tuttlingen; Germany): The plasma fractions were stored on ice until analysis or were frozen at –20 °C until analysis. All plasma samples were labelled with the following data:

- Code number
- Animal Number
- Date of sampling
- Time of sampling

Analytical Methods

The assay mixture for determination of plasma DPIV activity consisted of 80 µl reagent and 20 µl plasma sample. Kinetic measurement of the formation of the yellow product 4-nitroaniline from the substrate glycylprolyl-4-nitroaniline was performed at 390 nm for 1 min at 30 °C after 2 min pre-incubation at the same temperature. The DPIV activity was expressed in mU/ml.

Statistical methods

Statistical evaluations and graphics were performed with PRISM® 3.02 (GraphPad Software, Inc.). All parameters were analysed in a descriptive manner including mean and SD.

6.1 Results – In vivo DPIV-inhibition at t_{max}

Compound	Dose (mg/kg)	i.v. (%)	p.o. (%)
H-Ala-Val-Pro-CH_2O-CO-CH_3*HBr	100	89	87
H-Ala-Val-Pro-CO-CH_2O-CO-Ph*HBr	100	95	68

Example 7: The effect of substituted amino ketones on glucose tolerance in diabetic Zucker rats

Study Design

ANIMALS

N=30 male Zucker rats (fa/fa), mean age 11 weeks (5-12 weeks), mean body weight 350 g (150-400 g), were purchased from Charles River (Sulzfeld, Germany). They were kept for >12 weeks until all the fatty Zucker rats had the characteristics of manifest Diabetes mellitus.

HOUSING CONDITIONS

Animals were kept single-housed under conventional conditions with controlled temperature (22±2 °C) on a 12/12 hours light/dark cycle (light on at 06:00 a.m.). Standard pellets (ssniff®, Soest, Germany) and tap water acidified with HCl were allowed ad libitum.

CATHETERIZATION OF CAROTID ARTERY

Fatty Zucker rats, 17-24 weeks old, adapted to the housing conditions, were well prepared for the tests. Catheters were implanted into the carotid artery of fatty Zucker rats under general anaesthesia (i.p. injection of 0.25 ml/kg b.w. Rompun® [2 %], BayerVital, Germany and 0.5 ml/kg b.w. Ketamin 10, Atarost GmbH & Co., Twistringen, Germany). The animals were allowed to recover for one week. The catheters were flushed with heparin-saline (100 IU/ml) three times per week.
In case of catheter dysfunction, a second catheter was inserted into the contralateral carotid artery of the respective rat. After one week of recovery from surgery, this animal was reintegrated into the study. In case of dysfunction of the second catheter, the animal was withdrawn from the study. A new animal was recruited and the experiments were continued in the planned sequence, beginning at least 7 days after catheter implantation.

EXPERIMENTAL DESIGN

Fatty Zucker rats with intact catheter function were given in random order placebo (1 ml saline, 0.154 mol/l; N=9 animals as control), or test substance, solved in 1 ml saline (N=6 animals in each test group).
After overnight fasting, the fatty Zucker rats were given placebo and test substance, respectively, via feeding tube orally (15 G, 75 mm; Fine Science Tools, Heidelberg, Germany) at –10 min. An oral glucose tolerance test (OGTT) with 2 g/kg b.w. glucose as a 40 % solution (B. Braun Melsungen, Melsungen, Germany) was implemented at ±0 min. The glucose was administered via a second feeding tube. Arterial blood samples from the carotid catheter were collected at –30 min, -15 min, ±0 min and at 5, 10, 15, 20, 30, 40, 60, 90 and 120 min into 20 µl glass capillaries, which were placed in standard tubes filled with 1 ml solution for hemolysis (blood glucose measurement).
In addition, arterial blood samples were taken at –30 min, at 20, 40 60 and 120 min from the carotid catheter of the conscious unrestrained fatty Zucker rats and given into ice cooled Eppendorf tubes (Eppendorf-Netheler-Hinz, Hamburg, Germany) filled with 10 µl sodium citrate buffer (pH 3.0) for plasma DP activity measurement.

Eppendorf tubes were centrifuged immediately (12000 rpm for 2 min, Hettich Zentrifuge EBA 12, Tuttlingen; Germany): The plasma fractions were stored on ice until analysis.

ANALYTICAL METHODS

Blood glucose: Glucose levels were measured using the glucose oxidase procedure (Super G Glukosemeßgerät; Dr. Müller Gerätebau, Freital, Germany).

The compounds of the present invention, tested in the *in vivo* assay, improved significantly the glucose tolerance after oral administration during an OGTT in Zucker rats (see 7.1).

7.1 Results – Improvement of glucose tolerance after administration of substituted amino ketones during an OGTT in Zucker rats

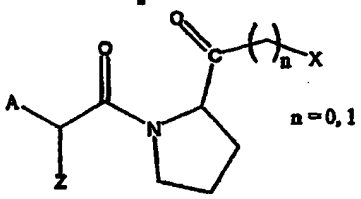
Compound	Dose (mg/kg b.w.)	Route of adm.	AUC Control (mmol*min/l)	AUC test compound (mmol*min/l)	Improve-ment (%)
H-Ala-Val-Pro-Me*HBr	100	oral	766.2	394.4	48.5
H-Val-Pro-Val-Pro-Me*HBr[1]	100	oral	118.8	66.4	44.1
H-Ala-Val-Pro-CH_2O-CO-CH_3*HBr	5	oral	561.5	309.1	44.9
H-Ala-Val-Pro-CH_2O-CO-CH_3*HBr	15	oral	561.5	300.9	46.4
H-Ala-Val-Pro-CH_2O-CO-CH_3*HBr	50	oral	561.5	254.7	54.6
H-Ala-Val-Pro-CO-CH_2O-CO-Ph*HBr	5	oral	517.3	209.1	59.5
H-Ala-Val-Pro-CO-CH_2O-CO-Ph*HBr	15	oral	517.3	245.4	52.6
H-Ala-Val-Pro-CO-CH_2O-CO-Ph*HBr	50	oral	517.3	160.5	69.0

[1] tested in Wistar rats under identical experimental conditions

Patent Claims

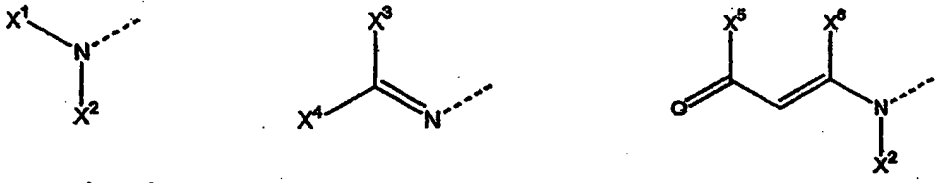
1. Compounds of the general formula 1

1



and pharmaceutically acceptable salts thereof, wherein:

A is selected from the following structures:



wherein

X^1 is H or an acyl or oxycarbonyl group including an amino acid residue, a N-protected amino acid residue, a peptide residue or a N-protected peptide residue,

X^2 is H, $-(CH)_m-NH-C_5H_3N-Y$ with m = 2-4 or $-C_5H_3N-Y$ (a divalent pyridyl residue) and Y is selected from H, Br, Cl, I, NO_2 or CN,

X^3 is H or selected from an alkyl-, alkoxy-, halogen-, nitro-, cyano- or carboxy- substituted phenyl or from an alkyl-, alkoxy-, halogen-, nitro-, cyano- or carboxy- substituted pyridyl residue,

X^4 is H or selected from an alkyl-, alkoxy-, halogen-, nitro-, cyano- or carboxy- substituted phenyl or from an alkyl-, alkoxy-, halogen-, nitro-,

cyano- or carboxy- substituted pyridyl residue,

X^5 is H or an alkyl, alkoxy or phenyl residue,

X^6 is H or an alkyl residue,

for n = 1

X is selected from: H, OR^2, SR^2, NR^2R^3, $N^+R^2R^3R^4$, wherein:

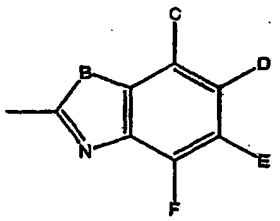
R^2 stands for acyl residues, which are optionally substituted with alkyl, cycloalkyl, aryl or heteroaryl residues, or for amino acid residues or peptidic residues, or alkyl residues, which are optionally substituted with alkyl, cycloalkyl, aryl or heteroaryl residues,

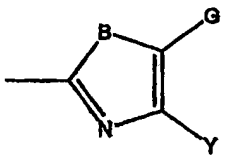
R^3 stands for alkyl or acyl residues, wherein R^2 and R^3 may be part of a saturated or unsaturated carbocyclic or heterocyclic ring,

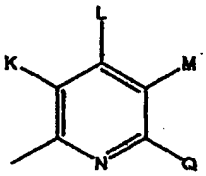
R^4 stands for alkyl residues, wherein R^2 and R^4 or R^3 and R^4 may be part of a saturated or unsaturated carbocyclic or heterocyclic ring,

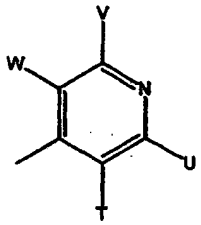
for n = 0

X is selected from:









wherein

B stands for: O, S or NR^5, wherein R^5 is H, alkyl or acyl,

C, D, E, F, G, Y, K, L, M, Q, T, U, V and W are independently selected from alkyl and substituted alkyl residues, oxyalkyl, thioalkyl, aminoalkyl, carbonylalkyl, acyl, carbamoyl, aryl and heteroaryl residues, and

Z is selected from H, or a branched or straight chain alkyl residue from C_1-C_8, a branched or straight chain alkenyl residue from C_2-C_8, a cycloalkyl residue from C_3-C_8, a cycloalkenyl residue from C_5-C_7, an aryl or heteroaryl residue, or a side chain selected from all side chains of all natural amino acids or derivatives thereof.

2. A compound according to claim 1, selected from the group consisting of 2-Methylcarbonyl-1-N-[(L)-Alanyl-(L)-Valinyl]-(2S)-pyrrolidine hydrobromide; 2-Methyl)carbonyl-1-N-[(L)-Valinyl-(L)-Prolyl-(L)-Valinyl]-(2S)-pyrrolidine hydrobromide; 2-[(Acetyl-oxy-methyl)carbonyl]-1-N-[(L)-Alanyl-(L)-Valinyl]-(2S)-pyrrolidine hydrobromide; 2-[Benzoyl-oxy-methyl)carbonyl]-1-N-[{(L)-Alanyl]-(L)-Valinyl]-(2S)-pyrrolidine hydrobromide; 2-[[(2,6-Dichlorbenzyl)thiomethyl]carbonyl]-1-N-[{(L)-Alanyl]-(L)-Valinyl]-(2S)-pyrrolidine; 2-[Benzoy-loxy-methyl)carbonyl]-1-N-[Glycyl-(L)-Valinyl]-(2S)-pyrrolidine hydrobromide; 2-[([1,3]-thiazole-2-yl)carbonyl]-1-N-[{(L)-Alanyl]-(L)-Valinyl]-(2S)-pyrrolidine trifluoracetat; 2-[(benzothiazole-2-yl)carbonyl]-1-N-[N-{(L)-Alanyl]-(L)-Valinyl]-(2S)-pyrrolidin trifluoracetat; 2-[(-benzothiazole-2-yl)carbonyl]-1-N-[{(L)-Alanyl]-Glycyl]-(2S)-pyrrolidine trifluoracetat; 2-[(pyridin-2-yl)carbonyl]-1-N-[N-{(L)-Alanyl]-(L)-Valinyl]-(2S)-pyrrolidine trifluoracetat.

3. A pharmaceutical composition for parenteral, enteral or oral administration, characterised in that it contains at least one compound according to any one of the preceding claims optionally in combination with customary carriers or excipients.

4. The use of compounds or pharmaceutical compositions according to any one of the preceding claims for the preparation of a medicament for the *in vivo* inhibition of DP IV or/and DP IV-like enzymes.

5. The use of compounds or pharmaceutical compositions according to any one of claims 1 to 3 for the preparation of a medicament for the treatment of diseases of mammals that can be treated by modulation of the DP IV activity of a mammal.

6. The use according to claim 5 for the treatment of metabolic diseases of humans.

7. The use according to claims 5 or 6 for the treatment of impaired glucose tolerance, glucosuria, hyperlipidaemia, metabolic acidosis, diabetes mellitus, diabetic neuropathy and nephropathy and of sequelae caused by diabetes mellitus, neurodegenerative diseases and disturbance of signal action at the cells of the islets of Langerhans and insulin sensitivity in the peripheral tissue in the postprandial phase of mammals.

8. The use according to claims 5 or 6 for the treatment of metabolism-related hypertension and cardiovascular sequelae caused by hypertension in mammals.

9. The use according to claims 5 or 6 for the prophylaxis or treatment of skin diseases and diseases of the mucosae, autoimmune diseases and inflammatory conditions.

10. The use according to claims 5 or 6 for the treatment of psychosomatic, neuropsychiatric and depressive illnesses, such as anxiety, depression, sleep disorders, chronic fatigue, schizophrenia, epilepsy, nutritional disorders, spasm and chronic pain.

11. The use according to claims 5 or 6 for the chronic treatment of chronic metabolic diseases in humans.

12. The use according to claims 5 or 6 for the chronic treatment of chronically impaired glucose tolerance, chronic glucosuria, chronic hyperlipidaemia, chronic metabolic acidosis, chronic diabetes mellitus, chronic diabetic neuropathy and nephropathy and of chronic sequelae caused by diabetes mellitus, chronic neurodegenerative diseases and chronic disturbance of signal action at the cells of the islets of Langerhans and chronic insulin sensitivity in the peripheral tissue in the

postprandial phase of mammals.

13. The use according to claims 5 or 6 for the chronic treatment of metabolism-related hypertension and of chronic cardiovascular sequelae caused by chronic hypertension in mammals.

14. The use according to claims 5 or 6 for the chronic treatment of chronic psychosomatic, chronic neuropsychiatric and depressive illnesses, such as chronic anxiety, chronic depression, chronic sleep disorders, chronic fatigue, chronic schizophrenia, chronic epilepsy, chronic nutritional disorders, spasm and chronic pain.

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(74) Agents: FORSTMEYER, Dietmar et al.; Boeters & Bauer, Bereiteranger 15, 81541 München (DE).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

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Published:
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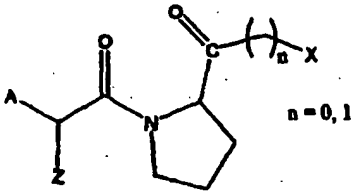
(54) Title: PEPTIDYL KETONES AS INHIBITORS OF DPIV



(I)

(57) Abstract: The present invention relates to compounds of the general formula (I), and pharmaceutically acceptable salts thereof, to the use of the compounds for the treatment of impaired glucose tolerance, glucosuria, hyperlipidaemia, metabolic acidosis, diabetes mellitus, diabetic neuropathy and nephropathy and of sequelae caused by diabetes mellitus in mammals.

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 02/08929

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K5/06 C07K5/08 A61K38/05 A61K38/06 A61P25/00
A61P3/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, CHEM ABS Data, MEDLINE, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 67279 A (SCHMIDT JOERN ;GLUND KONRAD (DE); DEMUTH HANS ULRICH (DE); HOFFMAN) 29 December 1999 (1999-12-29) page 11, paragraph 1.1 -page 17, last line; claims; table 1	1,3-8, 11,12
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A	US 4 643 991 A (DIGENIS GEORGE A ET AL) 17 February 1987 (1987-02-17) column 2, line 25 -column 6, line 34; claims; examples	1-12

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance
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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 December 2003	19. 01. 04

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Döpfer, K-P

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 468 469 A (JAPAN TOBACCO INC ;YOSHITOMI PHARMACEUTICAL (JP)) 29 January 1992 (1992-01-29) page 3, line 8 -page 6, line 9 page 34, line 55 -page 38, line 56 claims; tables	1-12
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X	TSUTSUMI SEIJI ET AL: "Synthesis and structure-activity relationships of peptidyl alpha-keto heterocycles as novel inhibitors of prolyl endopeptidase" JOURNAL OF MEDICINAL CHEMISTRY, vol. 37, no. 21, 1994, pages 3492-3502, XP002265524 ISSN: 0022-2623 Compounds: 7a,b,c; 12a,b; 14a; 20 tables 1,2	1,3-5,7, 10,14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 02/08929

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-12 (all partially)

   Peptidylketones of the general formula AZCH-CO-Pro-CH2-X (with X=N(+)R'R"R'", e.g. pyridinium), pharmaceutical compositions containing said methylketones and their use as medicaments

2. Claims: 1-12 (all partially)

   Peptidylketones of the general formula AZCH-CO-Pro-CH2-X (with X=OAcyl, SAcyl), pharmaceutical compositions containing said methylketones and their use as medicaments

3. Claims: 1-12 (all partially)

   Peptidylketones of the general formula AZCH-CO-Pro-CH2-X (with X=H), pharmaceutical compositions containing said methylketones and their use as medicaments

4. Claims: 1-12 (all partially)

   Peptidylketones of the general formula AZCH-CO-Pro-X (with X=2-thiazolyl, 2-benzthiazolyl, 2-pyridyl, 4-pyridyl), pharmaceutical compositions containing said methylketones and their use as medicaments

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 02/08929

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